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Role of the urokinase plasminogen activator system in responses to acute gastric mucosal injury and in *Helicobacter* infection

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Abstract

In common with other hollow organs, the gastric mucosa consists of exocrine and endocrine epithelial cells and sub-epithelial stromal cells, all of which secrete mediators into the tissue microenvironment that define mucosal architecture and maintain organ function. Gastric myofibroblasts are an important stromal cell population involved in maintaining mucosal integrity, and are implicated in pathophysiological processes including chronic inflammation, fibrosis and cancer. Urokinase plasminogen activator (uPA) and its inhibitor plasminogen activator inhibitor (PAI)-1 are secreted by gastric epithelial and stromal cells and, together with the uPA receptor (uPAR), are believed to be involved in epithelial-mesenchymal signalling. However, the role of the uPA system in regulating gastric mucosal morphological homeostasis and responses to acute and chronic challenges is not yet fully understood.

In this thesis, it was shown that transgenic PAI-1-H/Kβ mice, which have increased expression of PAI-1 in gastric parietal cells, have elevated concentrations of circulating PAI-1 and develop age-dependent increases in corpus mucosal thickness, independent of changes in parietal cell and myofibroblast abundance. PAI-1 did not have a direct trophic effect on gastric epithelial cells, indicating that PAI-1 might act via an indirect mechanism to modulate gastric epithelial cell turnover.

In order to determine whether PAI-1 influences the gastric tissue microenvironment via myofibroblast gene expression, global transcript expression profiles of gastric myofibroblasts from wild-type and PAI-1 null (PAI-1$^{-/-}$) mice were compared.

Whole genome microarrays and subsequent validation by immunofluorescence indicated that mouse antral myofibroblasts are highly heterogeneous, including both desmin positive and desmin negative cells with distinct global transcript expression profiles. Furthermore, there was evidence that antral myofibroblasts displayed phenotypic plasticity *in vitro*, developing a neuroendocrine-like phenotype marked by expression of secretogranin-2. Plasticity may be functionally significant *in vivo*, supporting the role of myofibroblasts in wound healing.

The role of PAI-1 in gastric mucosal responses to chronic *Helicobacter* infection was investigated using the *H. felis* model in wild-type, PAI-1$^{-/-}$ and PAI-1-H/Kβ mice. Both absence of PAI-1 and increased expression of gastric PAI-1 were protective against *Helicobacter*-induced preneoplastic gastric histopathology. Responses to acute gastric mucosal injury were investigated using intragastric indomethacin administration. Gastric PAI-1 protected against the development of lesions, whilst exogenous PAI-1 exacerbated the development of indomethacin-induced gastric mucosal injury. Gastric uPA expression did not effect the development of lesions, whilst absence of uPAR tended to exacerbate lesion development.

Taken together, the data presented in this thesis suggest a broadly protective role of PAI-1 in the gastric mucosa. A common therapeutic strategy for the prevention of NSAID-induced gastric injury, ulcer complications and progression of *Helicobacter*-induced gastric preneoplasia might be emerging, aimed at specifically increasing PAI-1 bioavailability in the gastric mucosa.

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Abbreviations

AMC	7-amino-4-methylcoumarin
ANOVA	Analysis of variance
AP-1	Activator protein 1
ATM	Adjacent tissue myofibroblast
ATP	Adenosine triphosphate
bFGF	Basic fibroblast growth factor
BMP	Bone morphogenetic protein
BSA	Bovine serum albumin
CAF	Cancer-associated fibroblast
cag PAI	*cag* pathogenicity island
CagA	Cytotoxin-associated gene A
CAM	Cancer-associated myofibroblast
CCK	Cholecystokinin
cDNA	Complementary deoxyribonucleic acid
CGRP	Calcitonin gene-related peptide
COX	Cyclooxygenase
CRF	Corticotrophin releasing factor
DAPI	4’, 6-diamidino-2-phenylindole
DMEM	Dulbecco’s Modified Eagle’s Medium
DMSO	Dimethylsulfoxide
DNA	Deoxyribonucleic acid
DPP4	Dipeptidylpeptidase 4
DTT	Dithiothreitol
EC	Enterochromaffin

ECL	Enterochromaffin-like
ECM	Extracellular matrix
EDTA	Ethylenediaminetetraacetic acid
EdU	5-ethynyl-2'-deoxyuridine
EGF	Epidermal growth factor
EGFR	Epidermal growth factor receptor
ELISA	Enzyme-linked immunosorbent assay
EMT	Epithelial-to-mesenchymal transition
FAK	Focal adhesion kinase
FBS	Fetal bovine serum
GFP	Green fluorescent protein
Gly-Pro-AMC	Gly-Pro-7-amido-4-methylcoumarin hydrobromide
GPCR	G protein-coupled receptor
GPI	Glycosylphosphatidylinositol
GRP	Gastrin releasing peptide
GTP	Guanosine-triphosphate
H&E	Haematoxylin and eosin
H. felis	*Helicobacter felis*
H. pylori	*Helicobacter pylori*
HB-EGF	Heparin-binding epidermal growth factor
HBSS	Hanks' Balanced Salt Solution
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulphonic acid
HGF	Hepatocyte growth factor
HUVEC	Human umbilical vein endothelial cell
IFN	Interferon
IGF	Insulin-like growth factor

IGFBP	Insulin-like growth factor binding protein
IL	Interleukin
IM	Intestinal metaplasia
IP	Intraperitoneal
IQR	Interquartile range
JNK	c-Jun N-terminal kinases
LPS	Lipopolysaccharide
LRP	Low-density lipoprotein receptor-related protein
MAPK	Mitogen-activated protein kinase
MAS5.0	Microarray Suite 5.0
MMP	Matrix metalloproteinase
mRNA	Messenger ribonucleic acid
NF-κB	Nuclear factor kappa-light-chain-enhancer of activated B cells
NO	Nitric oxide
NSAID	Non-steroidal anti-inflammatory drug
NTM	Normal tissue myofibroblast
PAI-1	Plasminogen activator inhibitor type-1
PAI-2	Plasminogen activator inhibitor type-2
PBS	Phosphate buffered saline
PDGF	Platelet derived growth factor
PGE2	Prostaglandin E2
PI3K	Phosphatidylinositol-3-kinase
pNA	p-nitroaniline
qPCR	Quantitative polymerase chain reaction
Reg-1	Regenerating islet-derived-1

RNA	Ribonucleic acid
SEM	Standard error of the mean
Shh	Sonic hedgehog
SMA	Smooth muscle actin
SPEM	Spasmolytic polypeptide expressing metaplasia
TBS	Tris-buffered saline
TFF	Trefoil factor family
TFSS	Type IV secretion system
TGF	Transforming growth factor
Th	T helper cell
TIMP	Tissue inhibitor of metalloproteinase
TNF	Tumour necrosis factor
tPA	Tissue plasminogen activator
uPA	Urokinase plasminogen activator
uPAR	Urokinase plasminogen activator receptor
VacA	Vacuolating cytotoxin
VEGF	Vascular endothelial growth factor
VSMC	Vascular smooth muscle cell

CHAPTER 1

INTRODUCTION

1.1 Overview

The epithelial lining of hollow organs consists of both exocrine and endocrine cells, which secrete mediators facilitating responses to the luminal environment that support mucosal homeostasis and organ function. In the glandular stomach, epithelial cells secrete mediators involved in luminal nutrient sensing and mucosal defence. The best-characterised gastric endocrine mediator is gastrin, which was identified as a gastric acid secretagogue by Edkins in the early 20th century, following the work of Bayliss and Starling on establishing the endocrine concept (Bayliss & Starling, 1902; Edkins, 1905; Edkins, 1906).

In order to detect and appropriately respond to nutrients and noxious stimuli, a sophisticated luminal sensing system exists in the gastric mucosa. Given that the gastric lumen is a potentially hostile low pH and proteolytic environment, the gastric mucosa possesses robust intrinsic defence mechanisms, at least partly mediated by prostaglandins and gaseous messengers, to maintain tissue integrity (Wallace, 2008). This defensive network is up-regulated in response to ingested noxious agents in the lumen. Both nutrient sensing and mucosal defence is mediated by an array of autocrine, paracrine, endocrine and neuronal interactions, between epithelial cells and the various stromal cell types found within the mucosa, including fibroblast-like cells, immune cells and neurons (Dockray, 2003; Wallace, 2008).

The myofibroblast is a key mediator of communication between epithelial cells and underlying stromal cells throughout the gastrointestinal tract. Residing within the lamina propria, myofibroblasts are in close proximity to both epithelial cells and subepithelial stromal cell populations, relaying local signals between these compartments (Valentich & Powell, 1994; Powell *et al.*, 1999). Consequently, myofibroblasts have a critical role in epithelial-mesenchymal cross talk maintaining gastric mucosal integrity, and also in pathophysiological states, such as chronic inflammation, fibrosis and cancer.

The urokinase plasminogen activator (uPA) system is comprised of an extracellular protease (uPA), its receptor (uPAR) and its inhibitors plasminogen activator inhibitor type-1 and type-2 (PAI-1 and PAI-2). These proteins have important regulatory roles in tissue remodelling, via a plasmin-activated proteolytic cascade and via non-proteolytic mechanisms. Consequently, the uPA system is involved in tissue homeostasis, wound healing, chronic inflammation, fibrosis and cancer (Andreasen *et al.*, 2000; Smith & Marshall, 2010). Both parenchymal and mesenchymal cells express members of the uPA system in many tissues, including the stomach (Kaneko *et al.*, 2003; Kenny *et al.*, 2008). Therefore, these proteins directly influence epithelial-mesenchymal signalling in mucosal tissues.

The aim of this thesis was to examine the role of the uPA system in regulating epithelial-mesenchymal signalling in the stomach, with a particular focus on responses to non-steroidal anti-inflammatory drug (NSAID)-induced gastric mucosal injury and chronic *Helicobacter* infection.

1.2 Gastric mucosal morphology & physiology

1.2.1 Gastric epithelial morphology

Although there are similarities in the gross morphology of different parts of the gastrointestinal tract, there are nevertheless marked differences in mucosal structure in different regions. The mammalian stomach consists of two distinct glandular regions (the corpus and antrum) comprised of different types of gland, containing exocrine and enteroendocrine cells. The corpus consists of oxyntic glands, containing hydrochloric acid-secreting parietal cells and pepsinogen-secreting chief cells, as well as mucous-secreting cells, enteroendocrine cells and rare caveolated cells (Karam & Leblond, 1992; Figure 1.1). Enteroendocrine cells of the adult corpus mucosa include histamine-secreting enterochromaffin-like (ECL) cells, somatostatin-secreting D cells, ghrelin-secreting cells, termed X/A-like cells in rodents and P/D1 cells in humans, and serotonin-secreting enterochromaffin (EC) cells (Simonsson *et al.*, 1988; Date *et al.*, 2000; Rindi *et al.*, 2002; Rindi *et al.*, 2004). The antrum consists of pyloric glands, populated by mucous-secreting cells and enteroendocrine cells (Figure 1.1). Antral enteroendocrine cells include gastrin-secreting G cells, D cells, EC cells and a few ghrelin-secreting cells (Tzaneva, 2003; Rindi *et al.*, 2004). The gastric epithelium exits as a continually renewing monolayer, arranged into invaginated tubular units that form the gastric pits and glands (Lee, 1985a; Karam & Leblond, 1992).

Corpus glands consist of four regions: pit, isthmus, neck and base. Gastric pits are populated mainly by mucous-secreting pit cells. Within the isthmus is located the

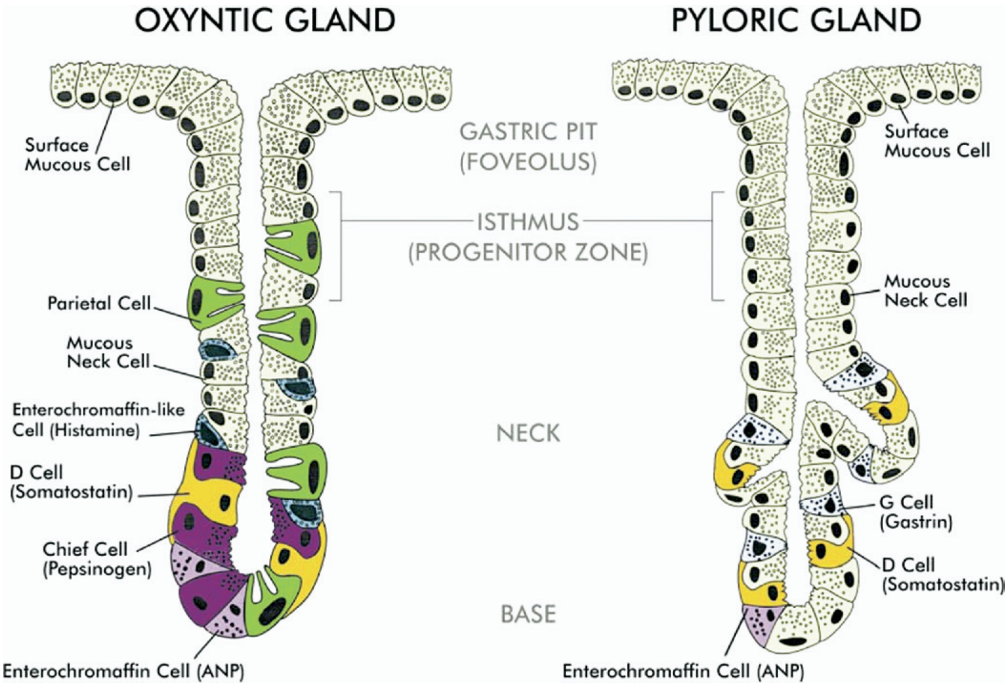

Figure 1.1. Major mature cell populations of the oxyntic and pyloric glands. The mature cell populations of the oxyntic gastric unit include surface mucous (pit) cells, parietal cells, mucous neck cells, chief cells and various enteroendocrine cells, including ECL cells, EC cells and D cells. Many mucous-secreting cells populate pyloric units, as well as enteroendocrine cells, including G cells, D cells and EC cells. Image reproduced with permission from Schubert & Peura, 2008.

granule-free stem cell and proliferating progenitor cells, from which all mature cell populations of the gastric unit are derived (Karam & Leblond, 1992). The neck region contains many mucous-secreting cells, whilst chief cells are restricted to the gland base. Parietal cells, enteroendocrine cells and caveolated cells are distributed along the length of corpus units (Karam & Leblond, 1992). Enteroendocrine cells are infrequent in gastric pits and increase in frequency from the isthmus and neck to the gland base (Karam & Leblond, 1993a). The morphology of the human corpus gastric unit is largely consistent with that of the mouse, except that mucous pit cells exclusively populate the pit and the stem cell contains mini-granules (Karam *et al.*, 2003).

Mucous-secreting cells are the principal cell population in antral units, exclusively populating the pit region and constituting much of the gland. Enteroendocrine cells are dispersed amongst the gland and isthmus regions, located within the basal third of the unit (Lee, 1985b; Lee & Leblond, 1985a; Lee & Leblond, 1985b). However, the isthmus is predominantly populated by the granule-free stem cell and proliferating progenitors.

1.2.2 Gastric epithelial stem cells

Experiments utilizing chimaeric mice or mutagenesis to trace cell lineage illustrate the monoclonal nature of adult mouse gastric units, whereby all differentiated cell types within the gastric unit can be traced to a single multipotential stem cell (Thompson *et al.*, 1990; Bjerknes & Cheng, 2002). It had been believed that all cells of the mouse antral unit were derived from the undifferentiated stem cell within the isthmus (Lee & Leblond, 1985a). However, an Lgr5-expressing stem cell has recently been identified within the basal region of some glands of the antrum, corpus-antrum transition zone and gastroesophageal junction in the adult mouse, and also within corpus glands of the developing mouse stomach (Barker *et al.*, 2010). Lineage tracing confirms that these stem cells are self-renewing and multipotential, generating epithelial cells for renewal of the gastric unit under basal conditions.

Identification of specific epithelial stem cell markers in the gastric mucosa is necessary for elucidating gastric stem cell biology. As well as the Lgr5-expressing population of stem cells at the gland base, a rare population of quiescent βgal-marked-villin expressing multipotential cells is located within or below the isthmus of some mouse antral glands (Qiao *et al.*, 2007). This particular population of stem cells is stimulated by pro-inflammatory signals, and may be important for epithelial restitution following mucosal injury. Phosphorylation of the specific linker Thr residues of Smad2/3 has been identified as a putative marker of quiescent stem cells in the mouse corpus and antral isthmus, representing stem cells in a pre-proliferative state under regulation by CDK4 and D-type cyclins (Fukui *et al.*, 2011). Recently, CD44 has been identified as a putative marker of the stem cell within the corpus unit, transducing proliferative signals responsible for basal stem cell turnover (Khurana *et al.*, 2013). However, much still remains to be elucidated about the molecular characteristics of gastric stem cells.

Various cell-types are believed to regulate the gastric epithelial stem cell niche. Parietal cells appear to be an important source of paracrine factors regulating stem cell and proliferating progenitor cell activity in corpus units, since their ablation leads to expansion of the granule-free stem cell, pit cell progenitor and mucous neck cell progenitor populations in mice (Li *et al.*, 1996). Subepithelial stromal cells are also believed to contribute to the maintenance of the epithelial stem cell niche. Myofibroblasts are secretory and contractile fibroblast-like stromal cells that also have characteristics of smooth muscle cells (Gabbiani *et al.*, 1971; Majno *et al.*, 1971; Powell *et al.*, 1999; Tomasek *et al.*, 2002). A more detailed description of the characteristics of myofibroblasts and a discussion on their definition can be found in section 1.3. Myofibroblasts are found within the lamina propria of gastric glands, and so are in close proximity to epithelial stem cells (Wu *et al.*, 1999). Their role in the regulation of the non-pathological gastric stem cell niche has not been elucidated but the role of stromal cells in maintaining the intestinal stem cell niche is better characterised. Proliferation of the intestinal stem cell is regulated by bone morphogenetic protein (BMP)-4 derived from subepithelial stromal cells, which suppresses Wnt signalling activity (He *et al.*, 2004). The colon cancer stem cell phenotype is maintained by myofibroblast-derived hepatocyte growth factor (HGF), activating Wnt signalling (Vermeulen *et al.*, 2010). Similar mechanisms may maintain the non-pathological gastric stem cell niche.

1.2.3 Gastric epithelial cell turnover and differentiation

In mouse corpus glands, undifferentiated stem cells undergo asymmetric division to generate three types of immature progenitor cells (pre-pit, pre-neck and pre-parietal cells), enteroendocrine cells and to renew themselves. Proliferating progenitors in the isthmus undergo bi-directional migration, depending upon the ultimate fate of the cell. Mutagenesis experiments have confirmed the existence of long-lived progenitors within the corpus isthmus (Bjerknes & Cheng, 2002). The proliferating pre-pit cell precursor gives rise to both pre-pit cells and pre-parietal cells with dense mucous granules. The proliferating pre-neck cell precursor yields pre-neck cells and pre-parietal cells with cored granules. A granule-free pre-parietal cell is also derived directly from the undifferentiated stem cell, as are enteroendocrine and caveolated cells (Karam & Leblond, 1993a; Karam & Leblond, 1993b).

Pre-pit cells have a turnover of less than 2.6 days and migrate upwards, towards the lumen (Karam & Leblond, 1993b; Karam & Leblond, 1993c). As they migrate upwards along the pit-axis, pre-pit cells mature into pit cells, developing larger and more numerous dense mucous secretory granules. Migration of pit cells along the pit axis takes approximately 3.1 days. Pre-neck cells have a turnover of approximately 3 days and migrate downwards, along the neck region, eventually becoming pre-zymogenic cells upon transitioning from the neck to the base region (Karam & Leblond, 1993b; Karam & Leblond, 1993d). Downwards migration of mucous neck cells along the neck axis takes approximately 1-2 weeks and is associated with maturation of the cored mucous secretory granules. Pre-zymogenic cells are marked by a replacement of mucous with pepsinogenic material in secretory granules, and maturation of zymogenic chief cells is associated with progressive replacement of mucous secretory granules with pepsinogenic granules. The turnover of the chief cell lineage is approximately 194 days.

Pre-parietal cells have a turnover of approximately 3 days and mature into parietal cells within the isthmus. Parietal cells migrate bi-directionally in the mouse corpus unit, to populate all sections of the gland and the pit (Karam, 1993; Karam & Leblond, 1993b). The turnover of parietal cells in these studies was reported as approximately 54 days, but a more recent study concluded that parietal cell turnover is in the region of 30-40 days (Kirton *et al.*, 2002). Upon reaching the pit surface or gland base, degenerated pit cells, chief cells and parietal cells either undergo apoptosis followed by phagocytosis by a neighbouring epithelial cell or macrophage or they undergo necrosis and are extruded (Karam, 1993; Karam & Leblond, 1993c; Karam & Leblond, 1993d).

Both granule-free and bi-directionally migrating immature granule-containing cells are found within the isthmus of the mouse antral gland (Lee & Leblond, 1985a). Antral mucous pit cells increase in maturity along the pit-axis from partially differentiated dense-granule cells at the pit-base, adjacent to the isthmus, becoming mature cells at the mid-pit region and senescing towards the luminal opening, where they are extruded or phagocytosed by adjacent cells (Lee, 1985b). Similarly, mucous gland cells increase in maturity during their downward migration from the isthmus-neck border, where they exist as immature core-granule cells, to the gland base, with extrusion or phagocytosis of cells occurring along the entire gland-axis (Lee & Leblond, 1985b).

Gastric enteroendocrine cells arise in the isthmus, differentiating directly from the granule-free stem cell (Lee & Leblond, 1985a; Karam & Leblond, 1993a). In the corpus, enteroendocrine cells migrate bi-directionally, but the majority migrate downwards, to populate the neck and the base of the gland with increasing frequency (Karam & Leblond, 1993a). All antral enteroendocrine cells migrate downwards (Lee & Leblond, 1985b).

1.2.4 Signalling pathways maintaining normal gastric epithelial cell turnover and differentiation in the adult stomach

Gastric mucosal morphology is maintained by autocrine, paracrine, endocrine and neuronal inputs, involving multiple epithelial and stromal cell-derived growth factors and signalling molecules. Parietal cells are key regulators of epithelial cell differentiation in the corpus unit. Deletion of parietal cells is associated with expansion of the pit-cell lineage and inhibition of the neck and chief cell differentiation sequence (Li *et al.*, 1996, Li *et al.*, 1998). Furthermore, targeted expression of simian virus 40 T antigen by pre-parietal cells results in loss of both the mature parietal cell and mature chief cell populations, owing to suppression of differentiation of pre-parietal and pre-zymogenic cells, and an expansion of the pre-parietal and pit cell populations (Li *et al.*, 1995). In another model of parietal cell ablation, tamoxifen-induced parietal cell loss is associated with the emergence of a spasmolytic polypeptide expressing metaplasia (SPEM)-like phenotype, characterised by a marked increase in proliferating stem and precursor cells, mucosal hypertrophy and expansion of cells expressing mucous neck cell markers into the gland base, indicating altered differentiation of chief cells (Huh *et al.*, 2012).

1.2.4.1 Epidermal growth factor receptor signalling

The epidermal growth factor receptor (EGFR) is expressed on mucous-secreting cells of the pit and gland regions of corpus and antral units, including the proliferative zone, on parietal cells and on G cells (Orsini *et al.*, 1993; Murayama *et al.*, 1995). Transforming growth factor (TGF)-α, an EGFR ligand, is expressed by differentiated epithelial cells of corpus and antral units, particularly by parietal cells and most robustly by pit cells (Thomas *et al.*, 1992; Abe *et al.*, 1997). Overexpression of TGF-α alters the differentiation sequence of corpus units. Phenotypically this is associated with mucosal hyperplasia and antralisation of the corpus unit, marked by expansion of the mucous pit cell population, loss of mature parietal and chief cells and migration of the proliferative zone towards the base of the gland (Sharp *et al.*, 1995; Goldenring *et al.*, 1996).

Other EGFR ligands expressed by gastric epithelial cells include heparin-binding epidermal growth factor (HB-EGF), which is expressed predominantly by parietal cells in the corpus unit and G cells in the antral unit, and amphiregulin, expressed by parietal cells (Murayama *et al.*, 1995; Abe *et al.*, 1997). Amphiregulin-deficient mice develop SPEM and mice expressing a mutant and defective EGFR develop SPEM more rapidly following parietal cell ablation (Ogawa *et al.*, 2006; Nam *et* *al.*, 2009). This further highlights the requirement of intact EGFR signalling and the role of the parietal cell population in the maintenance of normal turnover and differentiation of gastric epithelial cells.

1.2.4.2 Transforming growth factor-β signalling

BMPs are members of the TGF-β family of signalling proteins, and are expressed by both epithelial cells and myofibroblasts in the gastric mucosa (van den Brink *et al.*, 2001). BMP-4, expressed by myofibroblasts, is a downstream target of Sonic hedgehog (Shh). Noggin is an inhibitor of BMP signalling and its overexpression and subsequent suppression of gastric epithelial cell BMP signalling is associated with parietal cell loss and expansion of mucous neck cells, zymogenic cells, cells expressing markers of both of these cell types and the proliferating cell populations i.e. a SPEM-like phenotype (Shinohara *et al.*, 2010). This hyperproliferative state is associated with elevated expressions of amphiregulin and TGF-α. Conversely, BMP signalling negatively regulates proliferation of intestinal stem cells to maintain the quiescent stem cell niche, and may have a similar role in the stomach (He *et al.*, 2004).

Activins are signalling molecules of the TGF-β family known to have an important role in gastric epithelial cell differentiation. Activin receptor type II is expressed by parietal, pit and zymogenic cells within the gastric corpus and activation of signalling via this receptor by elevated concentrations of circulating activins results in loss of mature parietal cells, inhibition of zymogenic lineage differentiation and expansion of the granule-free, pit, pre-pit, caveolated and pre-caveolated cell populations, recapitulating some of the effects of parietal cell ablation (Li *et al.*, 1998). This is not associated with altered rates of proliferation or apoptosis however.

1.2.4.3 Insulin-Like Growth Factor signalling

Insulin-like growth factor (IGF)-I and IGF-II are secreted by myofibroblasts in the gastrointestinal mucosa, mediating mesenchymal-epithelial signalling via receptors on epithelial cells and stromal cells; IGF-I and IGF-II stimulate proliferation and migration of gastric myofibroblasts and gastric epithelial cells (Steenfos *et al.*, 1992; Powell *et al.*, 1999; Watanabe *et al.*, 2000; Hemers *et al.*, 2005; McCaig *et al.*, 2006). Expression of IGF-I, IGF-I receptor and IGF signalling molecules is enriched in the gastric epithelial progenitor cell population compared to adjacent pit cells, and it has been proposed that IGF signalling is a critical mediator of gastric epithelial progenitor cell biology, under regulation by pit-cell derived factors (Mills *et al.*, 2002).

1.2.4.4 Hedgehog Signalling

Shh is expressed by epithelial cells of the corpus gland and activates signalling in both epithelial and myofibroblast-like cells of the gland, but not pit cells (van den Brink *et al.*, 2001; Fukaya *et al.*, 2006). Hedgehog signalling-dependent differentiation of the pit cell lineage is mediated by expression of Indian hedgehog (Fukaya *et al.*, 2006). The gastric mucosa of mice lacking Shh is replaced by an intestinal mucosal phenotype, highlighting the importance of this morphogen in normal gastric epithelial differentiation (Ramalho-Santos *et al.*, 2000). Furthermore, inhibition of Shh signalling results in a marked increase in epithelial cell proliferation, expansion of the pit cell population, diminished neck and chief cell populations and inhibition of zymogenic lineage differentiation, mirroring the effects of parietal cell ablation (van den Brink *et al.*, 2001; Xiao *et al.*, 2010). This suggests a pivotal role for this signalling pathway in mediating parietal cell organizing activity in the corpus unit.

1.2.4.5 Wnt signalling

It is postulated that Wnt signalling mediates the maintenance of the gastric stem cell phenotype. Lgr5-expressing stem cells respond to Wnt-signals, as determined by Axin2 expression and stimulation by intraperitoneal R-spondin1 administration (Barker *et al.*, 2010). Furthermore, activation of Wnt signalling in gastric epithelial cells is associated with de-differentiation in the corpus and hyperproliferation in both corpus and antral mucosa (Radulescu *et al.*, 2013).

1.2.4.6 Notch signalling

There is evidence that the proliferating stem and progenitor cell phenotypes are also maintained by Notch signalling. Hes1 expressing proliferative cells are found within the isthmus of corpus and antral glands and inhibition of Notch signalling is associated with almost complete suppression of gastric epithelial cell proliferation *in vivo* (Kim & Shivdasani, 2011). Additionally activation of Notch signalling in parietal cells results in de-differentiation into multipotential stem or progenitor cells capable of regenerating entire gastric units.

1.2.5 The effects of gastrin on gastric mucosal morphology

G cells in the gastric antral mucosa secrete the hormone gastrin, which modulates the activity of parietal cells and ECL cells via activation of the cholecystokinin (CCK)-2 receptor (Schmitz *et al.*, 2001). Exogenous and endogenous gastrins have a trophic effect upon corpus mucosa, which is associated with expanded parietal and ECL cell populations (Crean *et al.*, 1969; Willems *et al.*, 1972; Hansen *et al.*, 1976; Johnson, 1977; Dembinski & Johnson, 1979; Ryberg *et al.*, 1990a; Ryberg *et al.*, 1990b; Wang *et al.*, 1996; Tsutsui *et al.*, 1997; Kidd *et al.*, 2000; Jain & Samuelson, 2006). However, prolonged hypergastrinaemia is associated with parietal cell and ECL cell loss and progression to gastric adenocarcinoma (Wang *et al.*, 2000).

In a number of studies, hypergastrinaemia has been associated with CCK2 receptor-dependent increases in basal gastric epithelial cell apoptosis and increased susceptibility to γ-radiation- and *Helicobacter*-induced apoptosis of gastric epithelial cells, partially reversible by expression of glycine-extended gastrin (Kidd *et al.*, 2000; Cui *et al.*, 2004; Cui *et al.*, 2006; Przemeck *et al.*, 2008). Conversely, other studies have indicated that both amidated gastrin and glycine-extended gastrin exert anti-apoptotic effects on isolated gastric epithelial cells and gastric cancer cells, and also that expression of anti-apoptotic proteins is increased in hypergastrinaemia-associated ECL cell carcinoid tumours (Konturek *et al.*, 2003; Pritchard *et al.*, 2008; He *et al.*, 2008). This highlights the role of the microenvironment and cell phenotype in determining the effects of gastrin upon gastric epithelial cell apoptosis.

Whilst gastrin has been shown to directly stimulate proliferation of ECL cells, a direct mitogenic effect on parietal cells has not been demonstrated (Jain & Samuelson, 2006). Gastrin induces the expression of growth factors by gastric epithelial cells, which may mediate some of its trophic activity. Exogenous gastrin increases the expression of HB-EGF and amphiregulin by parietal cells, whilst prolonged hypergastrinaemia induces expression of TGF-α and HB-EGF (Tsutsui *et al.*, 1997; Miyazaki *et al.*, 1999; Wang *et al.*, 2000; Dickson *et al.*, 2006). Additionally, gastrin induces expression of the growth factor regenerating islet-derived 1 (Reg-1), expressed by ECL cells in the gastric mucosa (Fukui *et al.*, 1998; Higham *et al.*, 1999; Ashcroft *et al.*, 2004; Steele *et al.*, 2007). Increased secretion of these growth factors into the gastric mucosal microenvironment is thought to stimulate proliferation of epithelial progenitor cell populations via paracrine interactions (Jain & Samuelson, 2006). Furthermore, the presence of CCK-2 receptors on some gastric epithelial progenitors suggests that gastrin may act directly to stimulate proliferation of these cells (Kazumori *et al.*, 2001).

1.2.6 Gastric mucosal luminal sensing

In order that gastrointestinal morphology and function are maintained, a complex mucosal sensory system exists, facilitating appropriate responses to the highly variable luminal environment. These responses are mediated via paracrine, endocrine, neural and immune interactions, disruption of which results in gastrointestinal pathophysiology. An important function of the gastrointestinal mucosa is to integrate luminal nutrient content with appropriate gut and systemic responses. In the intestine, various enteroendocrine and caveolated cells express G protein-coupled receptors (GPCRs), transporters and ion channels on the apical surface, which act as taste receptors and luminal nutrient chemoreceptors. Activation of these nutrient receptors elicits the basolateral secretion of an array of paracrine mediators, acting upon local cells and afferent nerve terminals, and gut hormones, which together target the gut, brain and peripheral tissues to facilitate digestion, nutrient absorption, satiety, glucose homeostasis and peripheral nutrient processing (Tolhurst *et al.*, 2012; Reimann *et al.*, 2012).

In the stomach, antral G cells sense luminal nutrients via GPCRs, such as the extracellular calcium-sensing receptor, which acts as an amino acid sensor as well as sensing increases in luminal calcium and pH (Conigrave *et al.*, 2000; Quinn *et al.*, 2004; Haid *et al.*, 2011). Activation of these chemoreceptors stimulates endocrine secretion of gastrin (Feng *et al.*, 2010). Gastrin primarily acts via CCK-2 receptors on ECL cells, promoting histamine secretion, to stimulate secretion of gastric acid by parietal cells (Tari *et al.*, 1997; Watson *et al.*, 2006; Schubert & Peura, 2008). An increase in luminal gastric acid stimulates secretion of somatostatin by D cells, which acts upon G cells, ECL cells and parietal cells to inhibit secretion of gastrin, histamine and gastric acid respectively, forming a self-regulating system via negative feedback (Watson *et al.*, 2006; Schubert & Peura, 2008; Figure 1.2).

Secretion by enteroendocrine cells is modulated by basolateral inputs such as growth factors, cytokines and neurotransmitters (Dockray, 2003). Gastrin releasing peptide (GRP) positively regulates gastrin and somatostatin secretion (Watson *et al.*, 2006). Proinflammatory cytokines stimulate gastrin secretion but inhibit somatostatin release, increasing gastric acid secretion as part of the host response to gastric bacterial infection (Dockray, 2003). Sensing of the luminal environment also takes place at the subepithelial level of the mucosa and within the submucosa and muscular layers. Intramural mechanoreceptive vagal afferent fibres sense gastric distension following a meal; CCK potentiates firing of these afferents, inhibiting food intake (Berthoud & Powley, 1992; Schwartz *et al.*, 1993). Mucosal and submucosal mechanisms responsible for the detection of and responses to potentially injurious agents in the gastric lumen are discussed in section 1.4.

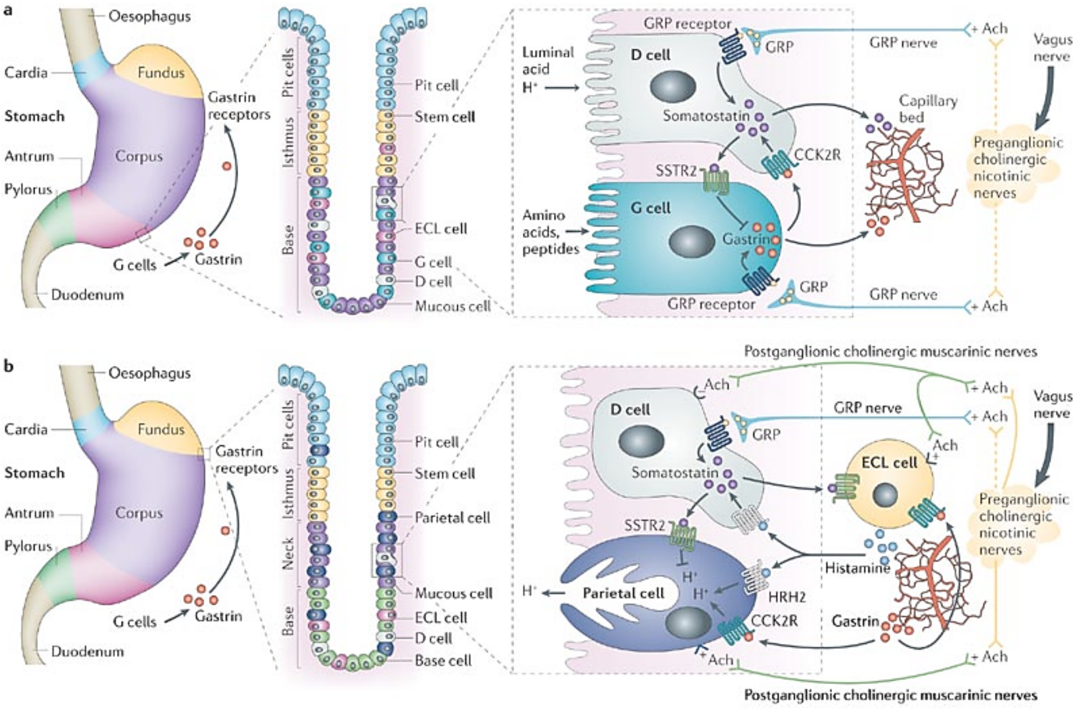

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Figure 1.2 Physiological control of gastric acid secretion. Following ingestion of a meal, nutrients, particularly amino acids, are sensed by antral G cells, stimulating endocrine secretion of gastrin. Gastrin acts via CCK2 receptors on ECL cells, potentiating histamine release, which subsequently stimulates exocrine secretion of gastric acid by parietal cells. Increases in luminal gastric acid stimulate somatostatin release from D cells, which acts to inhibit the secretion of gastrin, histamine and gastric acid, creating a self-regulating system. Image reproduced with permission from Watson *et al.*, 2006.

1.3 Myofibroblasts

1.3.1 Physiological and pathophysiological roles of myofibroblasts

Myofibroblasts are a key stromal cell population involved in physiological and pathophysiological tissue remodelling. During wound healing, myofibroblasts respond to tissue insult by migrating into the damaged tissue, where they produce paracrine factors involved in regulating epithelial responses, acute inflammatory responses, angiogenesis, neurogenic responses and extracellular matrix (ECM) remodelling (Powell *et al.*, 1999). The secretory and contractile myofibroblast produces ECM components, including fibronectin, laminins and particularly collagens, and transfers contractile force to granulation tissue to bring about wound contraction, before undergoing apoptosis (Mahida *et al.*, 1997; Tomasek *et al.*, 2002). Persistent activation of myofibroblasts in the contracted wound marks deregulation of ECM remodelling activity and tensional homeostasis, forming the basis of fibrosis in many tissues, including skin, lung, liver, kidney and heart (van den Borne *et al.*, 2010; Hinz *et al.*, 2012).

Myofibroblasts are also important mediators of immune responses, chronic inflammation and tumour progression. Myofibroblasts, fibroblasts and fibroblast-like cell populations act as antigen presenting cells and express Toll-like receptors, activation of which elicits secretion of cytokines, chemokines and other inflammatory mediators (Powell *et al.*, 2011; Pinchuk *et al.*, 2010). This contributes to persistent activation and recruitment of immune cells in chronically inflamed tissues (Andoh *et al.*, 2007; Pinchuk *et al.*, 2010; Pinchuk *et al.*, 2013). Myofibroblasts, fibroblasts and fibroblast-like cells can also function in an immunosuppressive role, in high interferon (IFN)-γ microenvironments (Powell *et al.*, 2011). Interactions between myofibroblasts in the tumour microenvironment and cancer cells drive invasive cancer cell growth, via direct cell-cell connections and paracrine interactions (De Wever *et al.*, 2008).

1.3.2 Morphological and molecular characterisation of myofibroblasts

Myofibroblasts are differentiated cells with characteristics of both fibroblasts and smooth muscle cells. Their discovery in granulation tissue led to the theory that the force required to elicit contraction during wound healing may be produced by these cells via the same mechanisms as smooth muscle contraction (Gabbiani *et al.*, 1971; Majno *et al.*, 1971). The myofibroblast is characterised by the presence of many mitochondria and cisternae of rough endoplasmic reticulum, as seen in the fibroblast, and obvious bundles of fibrils arranged in parallel with the cell axis, in keeping with smooth muscle like morphology. A basement membrane like material is observed on these cells, deposited in parallel to the cell surface at attachment sites, that can transfer contractile force generated within the cell to the ECM (Gabbiani *et al.*, 1971).

Vimentin and α-smooth muscle actin (SMA) are widely used as markers for differentiated myofibroblasts. Other markers of the differentiated myofibroblast may also include desmin, endosialin, podoplanin, osteopontin, periostin, fibroblast activation protein, palladin 4Ig, prolyl-4 hydroxylase, γ-SMA, P311, integrin α11β1, cadherin-11 and stromelysin-3, as well as absence of epithelial cytokeratins, smoothelin, CD14, CD31 and CD34 (Park *et al.*, 1999; De Wever *et al.*, 2008; Hinz *et al.*, 2012). Expression of these markers is variable and depends upon the tissue and cellular origin of the myofibroblast, which may derive from a number of cell types. Consequently, De Wever *et al.* have proposed that expression of α-SMA and at least three other markers is required to identify myofibroblasts as distinct from other myofibroblast-like cell populations, such as cancer-associated fibroblasts (CAFs) and activated fibroblasts (De Wever *et al.*, 2008). Similarly, Eyden has put forward more stringent criteria to define the myofibroblast, based on morphology, expression of protein markers and ultrastructural features (Eyden, 2008).

1.3.3 Myofibroblast lineages and differentiation

Transdifferentiation of fibroblasts is considered the primary source of myofibroblasts during epithelial wound healing, but myofibroblasts have also been suggested to arise from various undifferentiated and differentiated cell types, including mesenchymal stem cells, epithelial cells, endothelial cells, pericytes, myoepithelial cells, smooth muscle cells, fibrocytes, adipocytes, hepatic stellate cells, pancreatic stellate cells and mesothelial cells (De Wever *et al.*, 2008; Hinz *et al.*, 2012; Lv *et al.*, 2013). The various origins of a myofibroblast population depend upon the tissue and physiological or pathophysiological microenvironment they inhabit. It follows that myofibroblasts, which exist as phenotypically heterogeneous populations, can show characteristics of the parent cell type and it has been argued that there is some ambiguity in defining the myofibroblast in terms of its differentiation from other cells (Eyden, 2008). Phenotypically heterogeneous myofibroblasts, derived from different progenitors, may be functionally distinct from each other and may contribute different elements of the mechanisms required for their tissue remodelling activity in wound healing, fibrosis, chronic inflammation and tumourigenesis.

Fibroblast transdifferentiation occurs in response to paracrine factors, notably TGF-β, but possibly also platelet derived growth factor (PDGF), connective tissue growth factor, Wnt, Jagged, Shh, Fizz1, endothelin-1, lysophosphatidic acid and chemokines, produced by local inflammatory and parenchymal cells of the injured tissue (Werner & Grose, 2003; De Wever *et al.*, 2008; Liu *et al.*, 2009; Horn *et al.*, 2012; Hinz *et al.*, 2012). Treatment of cultured subcutaneous and lung fibroblasts with TGF-β induces expression of α-SMA, primarily through Smad2 phosphorylation (Desmouliere *et al.*, 1993; Evans *et al.*, 2003; Wipff *et al.*, 2007). Furthermore, administration of TGF-β to granulation tissue produces a distinct phenotype when compared to administration of other growth factors and cytokines: an abundance of myofibroblasts, which express α-SMA by definition, and collagenous deposits (Desmouliere *et al.*, 1993). TGF-β signalling via Smad synergises with hyaluronic acid-dependent co-localisation of EGFR and CD44 in lipid rafts, also stimulated by TGF-β signalling, to elicit fibroblast transdifferentiation (Midgley *et al.*, 2013). Stimulation of Wnt signalling in cultured mouse embryonic fibroblasts activates TGF-β signalling to induce α-SMA, via Smad2 phosphorylation, transforming the cells into contractile and migratory myofibroblasts (Carthy *et al.*, 2011). Conversely, TGF-β-induced transformation of skin fibroblasts into myofibroblasts is associated with activation of Wnt signalling and subsequent negative regulation of α-SMA expression (Liu *et al.*, 2012).

Fibroblast transdifferentiation is also stimulated by mechanical stimuli within the connective tissues, of which the fibroblast is a resident cell. When tissue is damaged, the ECM undergoes remodelling and loses its ability to shield resident cells from external forces and normal tissue tension. In response to exposure to these forces, fibroblasts express contractile stress fibres composed of cytoplasmic actins, the first step towards transformation into myofibroblasts (Tomasek *et al.*, 2002). Development of α-SMA-negative stress fibres is initiated only beyond a threshold ECM stiffness of ~3000-18000Pa in culture and *in vivo*, and the threshold for fully differentiated α-SMA-expressing myofibroblasts is further elevated to ~20000Pa in culture, translating to an ECM stiffness ~25000-50000Pa in contracting granulation tissue and fibrotic tissue (Hinz, 2010). ECM contraction activates latent TGF-β, via integrin-mediated mechanisms, thus myofibroblasts are able to stimulate further myofibroblast differentiation in granulation tissue and, significantly, during fibrosis (Wipff *et al.*, 2007). TGF-β signalling in fibroblasts is associated with disruption of microtubule polymerization and localization mDia2 to actin stress fibres, via Rho/SRF activation, facilitating stress fibre formation and myofibroblast differentiation (Sandbo *et al.*, 2013).

Bone marrow-derived myofibroblasts are myofibroblasts originating from circulating precursors, namely mesenchymal stem cells and CD34+ fibrocytes derived from CD34+ monocytes (De Wever *et al.*, 2008). Bone marrow derived myofibroblast precursors infiltrate tissues in response to chemokine signals, such as CXCL12 and SDF-1, which is particularly significant in myofibroblast recruitment to the tumour microenvironment (De Wever et al., 2008; Quante *et al.*, 2011). Bone marrow-derived myofibroblasts are also found in injured and fibrotic tissue stroma (Fujimiya *et al.*, 2009; Akita *et al.*, 2012; Binai *et al.*, 2012).

Epithelial cells are another potential source of myofibroblasts-like cells, via epithelial-to-mesenchymal transition (EMT). Resident epithelial cells lose epithelial markers and develop mesenchymal markers, including α-SMA. Activation of various signalling pathways induces EMT *in vitro* and *in vivo*, including Notch, hedgehog and Wnt, via integration with TGF-β signalling, (Zavadil *et al.*, 2004; Syn *et al.*, 2009; Aoyagi-Ikeda *et al.*, 2011; Chen *et al.*, 2012). Recently, bi-phenotypic cells, expressing markers of both epithelial cells and myofibroblasts, have been identified within the surface mucosa of human gastrointestinal biopsy tissues (Nemeth & Tiszlavicz, 2012). The origin of these cells remains to be elucidated, but their identification highlights the interchangeability of epithelial cell and myofibroblast phenotypes. In a manner similar to EMT, endothelial cells may undergo transition to a myofibroblast-like phenotype, and this can also contribute to the pathogenesis of fibrotic diseases and cancer (Piera-Velazquez *et al.*, 2011; Lin *et al.*, 2012).

1.3.4 Myofibroblasts in gastric physiology and pathophysiology

Myofibroblasts are located within the lamina propria of gastrointestinal mucosa (Wu *et al.*, 1999; Powell *et al.*, 2011). Myofibroblasts cultured from colonies arising from denuded gastric mucosa have been phenotypically confirmed using antibodies to α-SMA and vimentin at passages 1 and 8, showing preservation of the phenotype in prolonged culture (Wu et al., 1999). Characterisation of cultured gastric myofibroblasts by electron microscopy also identifies groups of microfilaments running lengthwise below the cell membrane (Wu et al., 1999). Myofibroblasts interact with epithelial cells and other stromal cells by direct cell-cell communication and via secretion of an array of mediators, including proteases, growth factors, cytokines, chemokines and lipid products (Valentich & Powell, 1994; Powell *et al.*, 1999). Thus myofibroblasts are critical regulators of gastrointestinal epithelial cell function, both in normal physiology and in pathophysiological processes, including gastroduodenal ulcer, *Helicobacter* infection and gastric cancer.

Myofibroblasts migrate from the gastric lamina propria through pores in the basement membrane of human gastric mucosa denuded of surface epithelial cells, in a model of gastric mucosal injury (Wu et al, 1999). The role of myofibroblasts in responses to gastric mucosal injury is described in section 1.4.6. As a source of growth factors, cytokines, chemokines and other paracrine mediators, myofibroblasts mediate epithelial and immune responses during *Helicobacter* infection (Fu *et al.*, 1999; Nakamura *et al.*, 2002; Kim *et al.*, 2004a; McCaig *et al.*, 2006; Pinchuk *et al.*, 2013). Myofibroblasts also play an important role in the microenvironment of gastric adenocarcinoma, as described in section 1.5.3.

1.4 Gastric mucosal defence and injury

1.4.1 Local gastric mucosal defence mechanisms

The gastric mucosa is constantly exposed to a variety of cytotoxic agents and noxious stimuli, including gastric acid, pepsin, ingested toxins, bacteria, bacterial products, extremes of osmolality as well as mechanical stress; in humans there may also be exposure to ethanol and damaging drugs such as NSAIDs. A complex system of luminal sensing and defensive mechanisms protects the integrity of the mucosa. One of the primary functions of this cytoprotective system is to maintain a near-neutral pH at the luminal surface of the epithelium. This is achieved via secretion of bicarbonate and mucus by surface epithelial cells, creating an adherent gel that acts as a neutralising and physical barrier against back-diffusion of acid and pepsin (Allen & Flemstrom, 2005). A more loosely adherent mucus layer is also present, which acts as a lubricant and protects the mucosa from the shear stresses generated by ingested particles (Atuma *et al.*, 2001). Furthermore, a hydrophobic surfactant layer of active phospholipids is present within the adherent mucus on the surface epithelium, forming an additional barrier against acid back-diffusion (Goddard *et al.*, 1990).

The intrinsic properties of the surface epithelial cells impart further protection on the gastric mucosa, providing a second line of defence when the bicarbonate-mucus-phospholipid barrier is breached. Gastric epithelial cells are highly resistant to proton diffusion across their apical membrane, making them resistant to acid-induced damage (Wallace, 2008). Superoxide dismutase scavenges superoxide produced during normal epithelial cell metabolism, protecting mucosal cells from oxidative stress-induced injury (Al-Jiboury & Kaunitz, 2012). When exposed to noxious stimuli or cytotoxic agents, gastric epithelial cells produce protective heat shock proteins and superoxide scavenging enzymes (Laine *et al.*, 2008; Al-Jiboury & Kaunitz, 2012). The resilience of surface epithelial cells is further enhanced by their rapid turnover rate of approximately 3 days and extrusion of degenerated cells (Karam & Leblond, 1993c; Wallace, 2008). Together, these mechanisms ensure that a robust continuous barrier is maintained. Furthermore, the surface epithelial cells are connected by tight junctions that enable modulation of paracellular permeability, forming a physical barrier against acid and pepsin back diffusion (Takezono *et al.*, 2004; Laine *et al.*, 2008).

Exposure of the surface epithelium to acid and other irritants stimulates a hyperaemic response, representing a third line of mucosal defence. Spinal afferent neurons innervating the mucosa detect acid entry into the mucosa via acid sensing channels within their terminals, located just beneath the surface epithelial cells, and also sense the luminal environment (Laine *et al.*, 2008). Activation of these neurons in response to noxious stimuli results in the release of calcitonin gene-related peptide (CGRP) from nerve terminals in close proximity to submucosal arterioles, resulting in generation of the vasodilator nitric oxide (NO) and consequently increased mucosal blood flow (Guth, 1992; Wallace, 2008; Laine *et al.*, 2008). This facilitates rapid dilution and removal of the noxious agent. Endothelial cells of the mucosal microvasculature also produce NO and other vasodilators, such as prostacyclin and hydrogen sulphide (Fiorucci *et al.*, 2006; Laine *et al.*, 2008). As well as maintaining microcirculatory blood flow, these agents attenuate inflammatory cytokine production and prevent leukocyte and platelet adherence to endothelial cells, thus protecting the microvasculature and preventing inflammatory cell extravasation into the mucosa (Guth, 1992; Wallace & Miller, 2000; Fiorucci *et al.*, 2006; Laine *et al.*, 2008).

1.4.2 Systemic gastric mucosal defence mechanisms

In addition to locally mediated mechanisms, central nervous system- and hormone-mediated mechanisms contribute to gastric mucosal defence. Direct and adaptive gastric cytoprotection is impaired in acutely vagotomised animals, rendering them more sensitive to mucosal injury in response to cytotoxic agents (Henagan *et al.*, 1984; Mozsik *et al.*, 1991; Evangelista & Maggi, 1991; Karadi et *al.*, 1999). Endogenous and exogenous CCK and leptin, administered centrally or peripherally, induce NO-mediated hyperaemia via a vago-vagal reflex involving activation of capsaicin-sensitive afferent fibres, independently of prostaglandin production (Evangelista & Maggi, 1991; Konturek *et al.*, 1995; Heinemann *et al.*, 1996, Brzozowski *et al.*, 2001). Central activation of the vagus nerve also increases surface epithelial cell pH and mucus viscosity.

Central administration of various other peptides protects against ethanol-induced gastric mucosal injury via vagal stimulation and subsequent release of CGRP, NO and, to varying degrees, prostaglandins. Centrally acting gastroprotective peptides include thyrotropin releasing hormone, corticotrophin releasing factor (CRF), peptide YY, ghrelin, orexin, glucagon-like peptide-1, enkephalins, β-endorphin, nociceptin, nocistatin, CGRP, calcitonin, adrenomedullin, amylin and cannabinoids (Brzozowski *et al.*, 2005; Shujaa *et al.*, 2009; Tache, 2012). Similarly, peripheral administration of ghrelin and orexin induces gastric cytoprotection, via stimulation of vagal-CGRP-NO-mediated hyperaemia and prostaglandin-mediated mechanisms (Konturek *et al.*, 2004; Brzozowski *et al.*, 2005; Brzozowski *et al.*, 2008).

Gastric cytoprotection is also elicited by peripheral administration of CRF, possibly through stimulation of gastroprotective glucocorticoids and also via activation of CRF receptor type 2 on gastric epithelial cells, inhibiting apoptosis (Chatzaki *et al.*, 2006; Filaretova *et al.*, 2012). Gastrin is gastroprotective via activation of CCK-2 receptors on afferent neurons, stimulating CGRP release and NO-mediated hyperaemia (Stroff *et al.*, 1995; Mercer *et al.*, 1997) Melatonin, produced by the pineal gland and enteroendocrine cells, stimulates a number of gastroprotective mechanisms, in part via an increase in plasma gastrin (Brzozowska *et al.*, 2009; Konturek *et al.*, 2010). Peripheral administration of peptide YY to plasma concentrations mimicking postprandial levels induces gastric cytoprotection via mechanisms independent of vagal stimulation (Kawakubo *et al.*, 2002).

1.4.3 Gastric mucosal defence against bacteria and bacterial products

Gastric cytoprotection extends to antibacterial mechanisms, to protect the mucosa against noxious interactions with ingested bacteria and bacterial products and prevent entry of bacteria and toxins into the circulation. Gastric secretions form the first line of defence. Gastric acid creates a hostile luminal environment for many bacteria, such that bactericidal activity of gastric juice is critically dependent upon a pH below 4 (Giannella *et al.*, 1972). Gastric juice contains antibacterial lysozyme, lactoferrin and immunoglobulins (Valnes *et al.*, 1984; Wallace, 2008). The mucus layer acts as a bacterial trap, aiding clearance of bacteria from the gastric lumen (Wallace, 2008). Together these mechanisms prevent bacterial colonisation of the gastric mucosa and subsequent damaging inflammatory responses.

The second line of defence against bacterial colonisation involves activation of innate immune responses. Toll-like receptors on gastric epithelial cells detect luminal bacteria, via recognition of pathogen-associated molecular patterns (Aderem & Ulevitch, 2000; Schmausser *et al.*, 2004). This activates signalling cascades that stimulate secretion of antibacterial peptides by epithelial cells, including cathelicidin and β-defensins (Cunliffe & Mahida, 2004; Laine *et al.*, 2008). Despite the robust antibacterial defence mechanisms that exist in the stomach, a number of species of bacteria have evolved mechanisms to evade destruction and clearance, notably some *Helicobacter* species.

1.4.4 Prostaglandins in gastric mucosal defence

Prostaglandins are products of arachidonic acid metabolism, catalysed by cyclooxygenase (COX) enzymes (Vane, 1971; Whittle, 1981). Constitutive production of prostaglandins is mediated via the COX-1 isoform, expressed in most tissues, whereas the COX-2 isoform is inducible and produces prostaglandins in response to inflammation (Kargman *et al.*, 1996; Peskar *et al.*, 2001). In the stomach, COX-1 and COX-2 are predominantly expressed by myofibroblasts, fibroblasts and macrophages (Wu *et al.*, 1999; Tatsuguchi *et al.*, 2000; Fu *et al.*, 1999; van Rees *et al*., 2002, Kim *et al.*, 2004a). Epithelial cells express COX-2 in gastric adenocarcinoma (van Rees *et al*., 2002). Both COX-1 and COX-2 activity contributes to prostaglandin-mediated gastric cytoprotection (Wallace *et al.*, 2000; Peskar *et al.*, 2001; Laine *et al.*, 2008). Simultaneous inhibition of COX-1 and COX-2 produces gastric mucosal injury, associated with a significant decrease in gastric mucosal prostaglandin E2 (PGE2) expression. Injury can be significantly attenuated by intravenous administration of PGE2 4 hours after administration of combined COX-1 and COX-2 inhibitors (Tanaka *et al.*, 2002).

Prostaglandins mediate many of the cytoprotective mechanisms in the gastrointestinal tract and are an essential component of the gastric mucosal defence system. Prostaglandins inhibit the secretion of gastric acid, accounting in part for their protective activity. However, gastric cytoprotection induced by exogenous prostaglandins is seen at concentrations below anti-secretory doses (Robert *et al.*, 1979). Secretion of bicarbonate, mucus and surfactant phospholipids is enhanced by prostaglandins, in favour of gastric mucosal defence (Wallace, 2008). Prostaglandins produced by the COX-1 isoform maintain normal mucosal blood flow and gastric motility, to protect the integrity of the microvasculature and prevent mucosal hypoxia (Wallace *et al.*, 2000; Laine *et al.*, 2008). Furthermore, prostaglandins attenuate deleterious inflammatory responses, such as mast cell, macrophage and neutrophil activation and adherence of leukocytes to the microvascular endothelium (Wallace *et al.*, 2000; Wallace, 2008). Prevention of leukocyte adherence is mediated by prostaglandins produced via the COX-2 isoform (Wallace *et al.*, 2000). Prostaglandins also act directly on epithelial cells to enhance their resilience to cytotoxic agents (Wallace, 2008). Furthermore, modulation of paracellular permeability in response to exposure of surface epithelial cells to acid is mediated by PGE2 (Takezono *et al.*, 2004).

1.4.5 NSAID-induced gastric mucosal injury

NSAIDs produce anti-inflammatory effects via systemic inhibition of COX-1 and COX-2 enzymes, preventing the production of prostaglandins (Vane, 1971; Whittle, 1981; Higgs *et al.*, 1987). Aspirin has a distinct mechanism of action compared to other NSAIDs, irreversibly inhibiting COX-1 via acetylation of the hydroxyl group of Ser 530, preventing access for arachidonic acid to the active site (Roth *et al.*, 1975). Consequently, prostaglandin-mediated gastric cytoprotective mechanisms are attenuated by NSAID administration, rendering the gastric mucosa more susceptible to injury by cytotoxic agents that the stomach is routinely exposed to (Wallace, 2008). NSAIDs also have direct topical cytotoxic effects on gastric epithelial cells and are also likely to produce gastric mucosal damage indirectly via some prostaglandin-independent mechanisms (Wallace, 2008; Laine *et al.*, 2008, Takeuchi, 2012). Upper gastrointestinal bleeding and ulceration is a common clinical consequence of NSAID treatment for this reason (Levy, 1974; Somerville *et al.*, 1986; Carson *et al.*, 1987; Hernandez-Diaz & Rodriguez, 2000).

1.4.6 Responses to gastric mucosal injury

Despite the robust mucosal defence system in the stomach, cytoprotective mechanisms are often breached, resulting in some level of gastric mucosal injury. The first level of mucosal injury involves loss of surface epithelial cells. In order to maintain the integrity of the epithelial barrier function and prevent deep mucosal injury, epithelial restitution occurs rapidly in response to denudation of the mucosa. Restitution is driven by migration of epithelial cells over periods of minutes to hours, followed over hours to days by proliferation in the isthmus (Lacy & Ito, 1984). These processes are differentially mediated by prostaglandins and a number of growth factors, including epidermal growth factor (EGF), TGF-α, HGF, keratinocyte growth factor, IGF-I, IGF-II, insulin and PDGF (Tarnawski *et al.*, 1985; Takahashi *et al.*, 1996; Tarnawski & Jones, 1998; Watanabe *et al.*, 2000; Tetreault *et al.*, 2005). EGFR is an important component of epithelial restitution mechanisms, transducing mitotic signals in response to direct activation by EGF or transactivation by prostaglandins (Tarnawski & Jones, 1998; Pai *et al.*, 2002). One mechanism by which NSAIDs induce gastric mucosal damage is by inhibiting EGFR signalling, impairing epithelial restitution (Pai *et al.*, 2001).

Epithelial restitution is also critically dependent upon the formation and maintenance of a protective mucoid cap over the exposed basement membrane, consisting of mucus, fibrin, plasma and cellular debris (Wallace & Whittle, 1986a; Wallace & Whittle, 1986b; Wallace & McKnight, 1990). The relatively high pH of

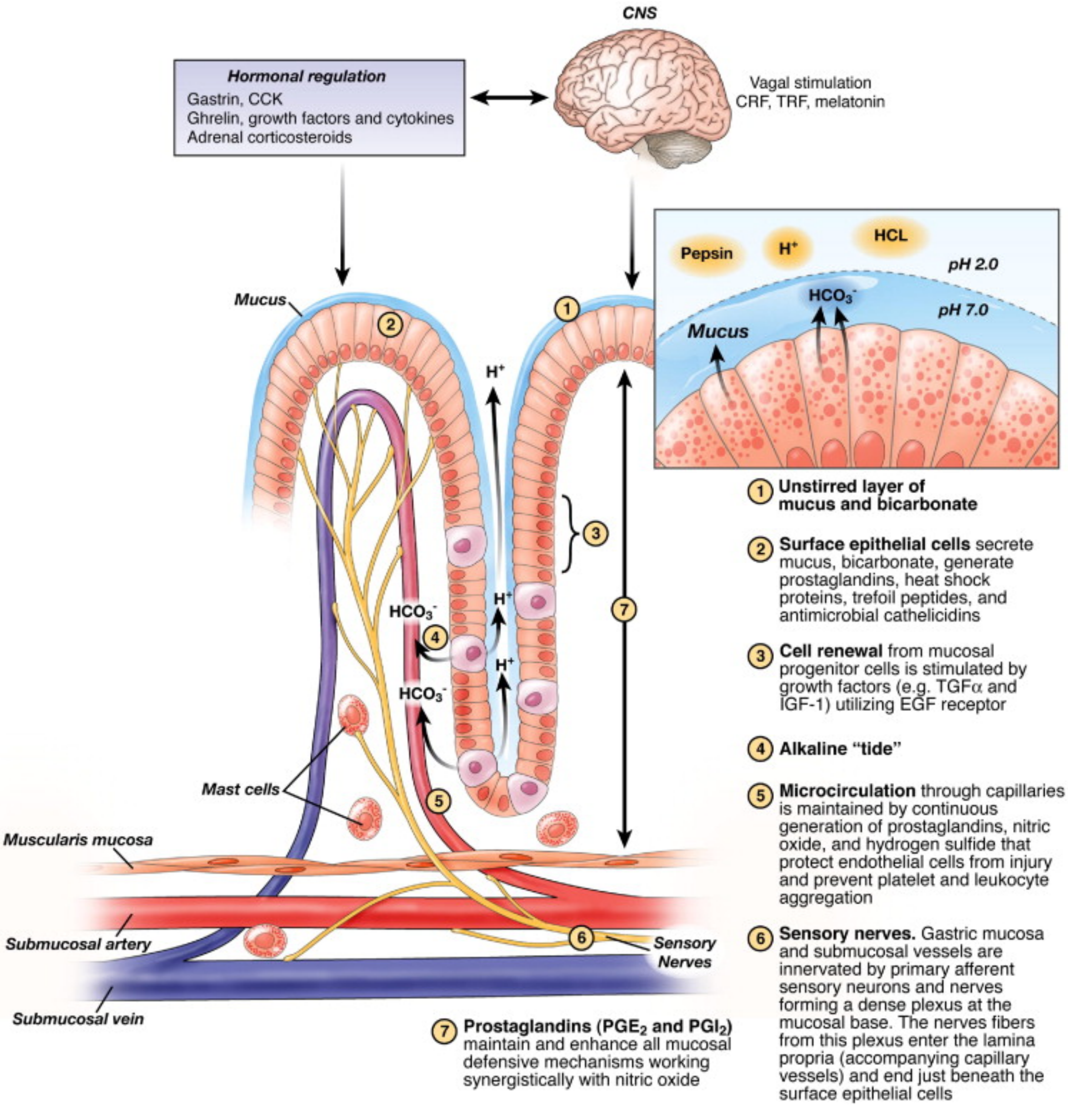


Figure 1.3. Gastric mucosal defence mechanisms can be triggered at multiple levels of the mucosa and submucosa, and are mediated via local and systemic signals. Image reproduced with permission from Laine *et al.*, 2008.

the mucoid cap is sustained by continuous mucosal blood flow; both NSAIDs and vasoconstricting agents, such as endothelin-1, disrupt the mucoid cap, facilitating deeper haemorrhagic mucosal injury (Wallace & McKnight, 1990). Trefoil factor family (TFF) peptides, secreted by gastric epithelial cells as a constituent of mucus, also enhance epithelial restitution, via modulation of epithelial cell migration and prevention of apoptosis of migrating cells (Hoffmann, 2005).

The next level of mucosal injury involves a breach of the basement membrane by cytotoxic agents, resulting in access to and necrosis of deeper mucosal layers, including endothelial cells of the microvasculature and epithelial cells of the gastric gland. This type of injury is characterised by gross haemorrhagic lesions and mucosal erosions. Gastric ulceration occurs when aggressive factors overcome defensive factors sufficiently to permit necrotic injury to penetrate the muscularis mucosae. Inflammatory responses to necrotic mucosal injury are characterised by activation of mast cells, macrophage and neutrophil recruitment and activation, adherence of leukocytes to the microvascular endothelium and extravasation of leukocytes and plasma into the mucosa, contributing to gastric mucosal injury (Martin & Wallace, 2006; Wallace, 2008). Ischaemia develops due to destruction of the microvasculature, release of vasoconstricting agents in response to inflammatory signals and thrombosis, augmenting mucosal necrosis (Tarnawski & Ahluwalia, 2012; Tarnawski *et al.*, 2012; Tarnawski *et al.*, 2013). Oxidative stress contributes to mucosal necrosis and gastric ulceration; consequently antioxidants are protective against gastric mucosal injury (Matsui *et al.*, 2011; Bindu *et al.*, 2011; Uc *et al.*, 2012).

Healing of necrotic mucosal lesions is a multi-step process, involving re-epithelialisation of surface and glandular mucosa, angiogenesis, to restore the disrupted microvasculature, and ECM remodelling, to rebuild the lamina propria (Tarnawski *et al.*, 2013). Healing of ulcers is more complex, involving extensive re-epithelialisation, angiogenesis and ECM remodelling, characterised by the formation of granulation tissue and eventual replacement with scar tissue (Wallace, 2008; Tarnawski & Ahluwalia, 2012; Tarnawski *et al.*, 2013). These regenerative processes are stimulated and mediated by various growth factors and signalling molecules, derived from epithelial cells, myofibroblasts, endothelial cells, platelets, inflammatory cells and bone marrow-derived mesenchymal stem cells (Wallace, 2008; Hayashi *et al.*, 2008; Tarnawski & Ahluwalia, 2012; Tarnawski *et al.*, 2012).

Re-epithelialisation occurs at the ulcer margin, to repopulate the necrotic area with mucosal glands and surface epithelial cells. Epithelial cell proliferation, migration and differentiation are stimulated by an array of signalling molecules, including EGF, Reg-1, IGF-I, HGF, PDGF, basic fibroblast growth factor (bFGF), TFF peptides, hedgehog signalling molecules, COX-2 derived prostaglandins and cytokines (Kang *et al.*, 2009; Fukuhara *et al.*, 2010; Tarnawski & Ahluwalia, 2012; Tarnawski *et al.*, 2013). As in restitution, EGFR is a critical mediator of gastric ulcer healing (Tarnawski & Jones, 1998; Tarnawski & Ahluwalia, 2012). Gastric glands develop from EGF secreting cells of the specialised ulcer-associated cell lineage, which invade granulation tissue, proliferate and migrate upwards from the ulcer base, forming tubular structures (Wright *et al.*, 1990). Additionally, bone marrow-derived mesenchymal stem cells are able to contribute to the regenerated epithelium (Okamoto *et al.*, 2002).

Angiogenesis arises within the granulation tissue, where endothelial cells proliferate and migrate from the intact microvessels surrounding the ulcer to form new capillaries and restore a microvascular network within the ulcer site (Tarnawski *et al.*, 2013). Microvascular tubulogenesis is stimulated by angiogenic growth factors, such as vascular endothelial growth factor (VEGF), bFGF, PDGF and angiopoietins (Tarnawski & Ahluwalia, 2012; Tarnawski *et al.*, 2013). Hypoxia-inducible factor-1, an inducer of VEGF expression, is produced in response to tissue ischaemia and consequently stimulates angiogenesis via VEGF (Ferrara, 2004). Similarly, VEGF expression is induced by various growth factors, melatonin, NO, cytokines and prostaglandins, produced in response to necrotic mucosal injury (Ferrara, 2004; Ganguly *et al.*, 2010; Tarnawski & Ahluwalia, 2012). TFF peptides also enhance angiogenesis, via COX-2 and EGFR signalling (Rodrigues *et al.*, 2003).

ECM remodelling takes place within ulcer granulation tissue, to provide a supportive matrix for the developing gastric glands and microvessels, and results in the formation of scar tissue. Myofibroblasts drive wound contraction and ECM deposition. Various growth factors, cytokines and signalling molecules drive migration and proliferation of fibroblasts and myofibroblasts, and myofibroblast differentiation, including TGF-β, PDGF, EGF, bFGF, IGF-I, tumour necrosis factor (TNF)-α, interleukin (IL)-1 and endothelin-1, derived from inflammatory cells and activated endothelial cells in granulation tissue (Watanabe *et al.*, 2000; Nishida *et al.*, 2006; Tarnawski & Ahluwalia, 2012). Extracellular proteases, such as matrix metalloproteinases (MMPs), are crucial in remodelling the ECM. Their expression and that of their inhibitors is tightly regulated during gastric ulcer healing, to facilitate appropriate degradation of ECM components for the healing process (Tarnawski, 2005).

1.5 *Helicobacter* infection and gastric adenocarcinoma

1.5.1 Epidemiology of *Helicobacter pylori*

H. pylori is a prevalent gastric bacterial pathogen, infecting up to 40% of the adult human population in developed countries, increasing to approximately 80-90% in developing countries (Rothenbacher & Brenner, 2003; Frenck & Clemens, 2003). Infection with *H. pylori* often goes undetected for a lifetime, as in the majority of cases infection causes mild, asymptomatic chronic pangastritis (McNamara & El-Omar, 2008). However, in some individuals, infection elicits pathogenic immune responses. Without treatment, approximately 10% of infected individuals develop peptic ulcer disease, associated with antral-predominant colonisation by the bacteria and acid hypersecretion (McColl *et al.*, 1997). Gastric adenocarcinoma develops in 1-3% of infected individuals and is associated with a corpus-predominant pattern of colonisation and acid hyposecretion, whilst <0.1% of infected individuals develop mucosa associated lymphoid tissue lymphoma (Uemura *et al.*, 2001; Peek & Blaser, 2002). A combination of host genetic background, expression of bacterial virulence factors and environmental stimuli determine the nature of interactions between the pathogen and host and thus the outcome of infection (Cover & Blaser, 2009).

H. pylori was first identified in 1982 as a pathogenic agent in peptic ulcer disease (Marshall & Warren, 1984). The Nobel Prize in Medicine was awarded to Robin Warren and Barry Marshall in 2005 for their discovery. A role in gastric carcinogenesis was subsequently recognised and given prominence when *H. pylori* was classified as a Group I human carcinogen in 1994 by the International Agency for Research on Cancer (IARC, 1994). As a consequence, *H. pylori* eradication has proven to be an effective strategy for reducing the incidence of gastric adenocarcinoma, in both humans and animal models (Leung *et al.*, 2004; Mera *et al.*, 2005; Peek & Crabtree, 2006; Fuccio *et al.*, 2007; Romero-Gallo *et al.*, 2008).

1.5.2 Characteristics of *Helicobacter pylori*

H. pylori is a gram-negative, microaerophilic, spiral-shaped and flagellate bacterium that colonises the gastric mucosa and is capable of interacting directly with host epithelial cells, by inhabiting the adherent mucus layer (Marshall & Warren, 1984; Schreiber *et al.*, 2004). Approximately 20% of the bacteria adhere to gastric epithelial cells (Peek & Crabtree, 2006). The bacteria are also able to penetrate into the mucosa, occupying both intracellular spaces within the gastric epithelial cells and interstitial spaces, including the lamina propria (Oh *et al.*, 2005; Necchi *et al.*, 2007; Ito *et al.*, 2008). Following ingestion, *H. pylori* rapidly migrates from the highly acidic stomach lumen to the relatively high pH niche of the adherent mucus layer. The bacteria utilise cytoplasmic urease to convert urea to ammonia in the acidic gastric environment. This facilitates colonisation by buffering the periplasm and altering the mechanical properties of the mucus, to make it more permissive to the flagellar and corkscrew motility of the bacteria (Weeks *et al.*, 2000; Salama *et al.*, 2013). Subsequently, *H. pylori* establish as the predominant bacterial species within the infected stomach (Cover & Blaser, 2009).

The presence of various virulence factor loci within the genome determines the pathogenic potential of different strains of *H. pylori.* Expression of virulence factors facilitates interactions with host cells, allowing manipulation of gene expression, cell signalling and the cytoskeletal machinery. *H. pylori* virulence factors include surface adhesins, vacuolating cytotoxin (VacA), γ-glutamyl transpeptidase, cytotoxin-associated gene A (CagA) and other gene products of the *cag* pathogenicity island (*cag* PAI) (Salama *et al.*, 2013). All known strains of *H. pylori* carry the *vacA* gene; expression of different isoforms determines the pathogenic potential of the protein in each strain (Atherton *et al.*, 1997; Basso *et al.*, 1998; Rhead *et al.*, 2007). VacA is a vacuolating and channel forming toxin that disrupts epithelial cell barrier function and polarity, induces apoptosis via mitochondrial targeting and attenuates host T-cell responses (Cover & Blanke, 2005; Palframan *et al.*, 2012).

H. pylori strains possessing the *cag* PAI interact with host epithelial cells via a type IV secretion system (TFSS), enabling direct translocation of CagA and other bacterial proteins into host cells (Odenbreit *et al.*, 2000; Backert *et al.*, 2000). Phosphorylation of translocated CagA induces epithelial cell proliferation, scattering and elongation, via binding and activation of SHP2 tyrosine phosphatase (Segal *et al.*, 1999; Higashi *et al.*, 2004; Rieder *et al.*, 2005; Tsutsumi *et al.*, 2006). CagA also acts via phosphorylation-independent mechanisms to increase epithelial cell proliferation and motility, as well as eliciting a number of cytoskeletal disturbances, disrupting epithelial cell polarity, increasing IL-8 secretion and inducing oxidative DNA damage and apoptosis (Crabtree *et al.*, 1995; Rieder *et al.*, 2005; Murata-Kamiya, 2011; Chaturvedi *et al.*, 2011). Consequently, CagA-expressing strains are associated with more severe host inflammatory responses, increased incidence of peptic ulcer disease and increased gastric cancer risk (Blaser *et al.*, 1995; Kuipers *et al.*, 1995; Parsonnet *et al.*, 1997; Matos *et al.*, 2013).

1.5.3 Gastric adenocarcinoma

Gastric cancer is the fourth most prevalent cancer and the second leading cause of cancer mortality worldwide (Parkin *et al.*, 2005). *H. pylori* infection is known to be a risk factor for both intestinal and diffuse types of gastric adenocarcinoma (Peek & Blaser, 2002). Intestinal-type adenocarcinoma develops through a multi-step process, arising on a background of corpus-predominant chronic gastritis, followed by multifocal glandular atrophy associated with hypochlorhydria, metaplasia, dysplasia and carcinoma (Correa, 1992; El-Omar *et al.*, 1997).

It has been suggested that parietal cell loss is induced by an autoimmune response to *Helicobacter* infection (Negrini *et al.*, 1997; Veijola *et al.*, 2010). Loss of parietal cells leads to SPEM and / or intestinal metaplasia (IM). In SPEM, loss of parietal cell-derived differentiation signals results in transdifferentiation of chief cells, so that they express markers of both chief and mucous cells (Weis & Goldenring, 2009). In mouse models of *Helicobacter* infection, dysplasia arises directly from the SPEM phenotype, driven by a T helper cell (Th)1-predominant immune response (Roth *et al.*, 1999; Nomura *et al.*, 2004). In humans, IM may arise from atrophic gastritis or the SPEM phenotype. IM is marked by replacement of the gastric epithelium with intestinal epithelial cell populations, such as absorptive cells, goblet cells and Paneth cells, owing to altered differentiation signals in the gastric mucosa (Leung & Sung, 2002). Progressive alterations in foveolar mucins also develop; acid sialomucins characteristic of the small intestine and finally colonic sulphated mucins are expressed (Silva *et al.*, 2002). Differential expression of mucins is seen in different IM sub-types (Reis *et al.*, 1999). Chronic *Helicobacter*-induced gastritis induces an accumulation of genetic mutations and epigenetic modifications, driving progression of metaplastic lesions through expansion of even a single clone of mutated glands (Jenks *et al.*, 2003; Kim *et al.*, 2004b; Gutierrez-Gonzalez *et al.*, 2011).

Stromal cell populations are important drivers of gastric carcinogenesis. Bone marrow derived stem cells can engraft into gastric tumour tissue, contributing to both myofibroblast and cancer cell populations (Houghton *et al.*, 2004; Worthley *et al.*, 2009). Myofibroblasts are able to drive invasive cancer growth by modulating the microenvironment (De Wever *et al.*, 2008). Gastric cancer-associated myofibroblasts (CAMs) undergo phenotypic changes compared to normal tissue myofibroblasts (NTMs) and adjacent tissue myofibroblasts (ATMs). These include increased migration and proliferation and a significantly altered secretome, stimulating increased proliferation, migration and invasion of cancer cells, in part via increases in secretion of proteases and increased IGF-II activity (Holmberg *et al.*, 2012; Fuyuhiro *et al.*, 2012; Holmberg *et al.*, 2013). Furthermore, CAMs from patients with poor survival lose the regulated secretory phenotype typical of gastric myofibroblasts (Balabanova, 2012). These alterations support the role of myofibroblasts as drivers of tumourigenesis, invasion and metastasis in gastric cancer.

1.5.4 *Helicobacter pylori* in the pathogenesis of gastric adenocarcinoma

Host inflammatory responses to *H. pylori* infection contribute strongly to the aetiology of gastric adenocarcinoma. Inflammatory responses stimulate an array of cancer-promoting activities including cell proliferation, transformation, migration, angiogenesis and inhibition of apoptosis (McNamara & El-Omar, 2008). *H. pylori* infection produces both lymphocytic and neutrophilic gastritis, which is determined by local inflammatory responses and activation of both innate and adaptive immune systems (Ernst & Gold, 2000; Robinson *et al.*, 2007).

Local release of pro-inflammatory cytokines and chemokines is stimulated via recognition of translocated bacterial peptidoglycan, by the Nod1 intracellular pattern recognition receptor, inducing nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) signalling in epithelial cells (Viala *et al.*, 2004). Secretion of cytokines and chemokines stimulates recruitment of neutrophils, monocytes and lymphocytes to the gastric mucosa (Ernst & Gold, 2000; Robinson *et al.*, 2007). Neutrophils induce oxidative DNA damage, contributing to the mutagenic effects of *Helicobacter* infection (Jenks *et al.*, 2003; Touati *et al.*, 2003). Another downstream effect of these local inflammatory signals relevant to carcinogenesis is the induction of proteases, such as uPA, MMP-7 and MMP-9, and their inhibitors, including PAI-1 and PAI-2 (Wroblewski *et al.*, 2003; Kitadai *et al.*, 2003; Varro *et al.*, 2004; Kenny *et al.*, 2008; Keates *et al.*, 2008). *H. pylori* also stimulates expression of a number of other genes implicated in promoting preneoplasia, metaplasia and carcinogenesis, including VEGF, HB-EGF, angiogenin and uPAR (Wallasch *et al.*, 2002; Kitadai *et al.*, 2003; Kenny *et al.*, 2008). In terms of adaptive immune responses, *H. pylori* induces a Th1-predominant response in humans, characterised by IFN-γ secretion and expression of pro-inflammatory cytokines such as TNF-α, IL-12, IL-2 and IL-18 (Robinson *et al.*, 2007; Wroblewski *et al*, 2010). This induces an aggressive inflammatory response, providing a background from which adenocarcinoma may arise (Sayi *et al.*, 2009).

Host genetic factors are important determinants of the pathological responses to *H. pylori* infection. Many polymorphisms exist within the array of pro- and anti-inflammatory cytokines that mediate gastritis in response to infection, so that there is a great deal of variation in the nature and magnitude of these responses amongst individuals. For instance, a number of studies have shown that polymorphisms in the pro-inflammatory IL-1 and TNF-α and anti-inflammatory IL-10 gene clusters increase the risk of cancerous and pre-cancerous lesions developing in response to *H. pylori* infection (McNamara & El-Omar, 2008).

Cancer-promoting epithelial cell and inflammatory responses are, in part, determined by the virulence of the infecting strain of bacteria. Many of the host inflammatory responses associated with *H. pylori*-induced pathologies are mediated by the TFSS, contributing to the increase in disease incidence associated with *cag* PAI-positive strains of the bacterium. Activation of NF-κB and subsequent induction of epithelial pro-inflammatory cytokines, such as IL-8, is dependent upon an intact TFSS, encoded by genetic loci on the *cag* PAI (Crabtree, 1998; Glocker *et al.*, 1998; Li *et al.*, 1999; Guillemin *et al.*, 2002). IL-8 is also induced by an active outer inflammatory protein A gene (Yamaoka *et al.*, 2000; Yamaoka *et al.*, 2002). Transcriptional responses of epithelial cells to infection with *cag* PAI-negative isogenic mutants resemble that of mock-infected cells, highlighting the importance of the TFSS for host cell responses to *H. pylori* (Guillemin *et al.*, 2002). Furthermore, epithelial cells infected with *cag* PAI-negative mutants differentially express an array of genes determining cell proliferation, apoptosis and transcriptional activity, as well as inflammatory cytokine and protease production, compared to *cag* PAI-positive wild-type bacteria (Cox et *al.*, 2001). CagA-stimulated modifications to the epithelial cell cytoskeleton and signalling pathways promote proliferation, migration and chromosome instability, enhancing preneoplastic remodelling and carcinogenesis (Segal *et al.*, 1999; Higashi *et al.*, 2004; Ohnishi *et al.*, 2008; Umeda *et al.*, 2009; Murata-Kamiya, 2011). Furthermore, evolution of *H. pylori* during progression from chronic atrophic gastritis to gastric adenocarcinoma results in a more invasive phenotype. Cancer-associated intracellular bacteria influence gastric epithelial stem cell activity in favour of carcinogenesis (Giannakis *et al.*, 2008).

1.5.5 Mouse models of *Helicobacter*-induced gastric carcinogenesis

A number of animal models of *Helicobacter* infection have been developed, to elucidate the mechanisms underlying host-bacterial interactions in gastric carcinogenesis. These utilise either adapted strains of *H. pylori* or alternative *Helicobacter* species, infected into mice or Mongolian gerbils for the most part, to recapitulate human pathology (Pritchard & Przemeck, 2004). The mouse is a particularly attractive animal model to use due its availability, relative low-cost to maintain compared to larger mammalian models and ease of genetic manipulation.

The genetic background of the host is an important determinant of the pathological outcome of infection, as illustrated by studies reporting varied responses of different mouse strains to infection with equivalent strains of *Helicobacter*

(Sakagami *et al.*, 1996; Mohammadi *et al.*, 1996; Lee *et al.*, 1997; Wang *et al.*, 1998; Kim *et al.*, 2001; Mahler e*t al.*, 2002; Thompson *et al.*, 2004). Host gender also influences responses to *Helicobacter* infection; female C57BL/6 mice develop more severe inflammatory and histopathological responses to *Helicobacter felis* infection (Court *et al.*, 2003). Conversely, male INS-GAS mice respond to *H. pylori* infection with an augmented histopathological response and accelerated progression to adenocarcinoma (Fox *et al.*, 2003). These opposing results also highlight the role played by *Helicobacter* species in shaping pathogenesis.

Cytotoxin producing strains of *H. pylori* elicit inflammatory and histological responses in mice with some features of those seen in humans (Marchetti *et al.*, 1995; Lee *et al.*, 1997). Adapted strains of *H. pylori* are developed by *in vivo* passage of clinical isolates with the highest colonisation efficiency, via serial gavage (Marchetti *et al.*, 1995; Lee *et al.*, 1997; Thompson *et al.*, 2004). One such strain is Sydney strain 1, used widely in mouse models owing to successful colonisation of the mouse gastric mucosa and persistent infection (Lee *et al.*, 1997). However, adaptation of *H. pylori* strains for effective colonisation of the mouse gastric mucosa results in diminished functionality of virulence genes, such as those of the *cag* PAI, eliciting an attenuated host response thus limiting true recapitulation of human pathogenesis (Philpott *et al.*, 2002; Court *et al.*, 2002; Crabtree *et al.*, 2002). Consequently, wild-type mice fail to develop gastric adenocarcinoma in response to *H. pylori* infection, in the absence of other carcinogenic agents (Lee *et al.*, 1997; Kim *et al.*, 2003; Pritchard & Przemeck, 2004).

H. felis is an alternative species of *Helicobacter* used in mouse models. Despite lacking the *cag* PAI, *H. felis* closely models human preneoplastic responses to *H.*

pylori in the stomach, when infected into the C57BL/6 mouse strain, including mucosal hyperplasia, oxyntic gland atrophy, glandular dilation, invasion of glands through the muscularis mucosae, lymphocytic infiltration, mucous cell metaplasia and SPEM, progressing to carcinoma in longer term infections (Fox *et al.*, 1996; Sakagami *et al.*, 1996; Fox *et al.*, 1997; Wang *et al.*, 1998; Lichtenberger *et al.*, 1999; Dial *et al.*, 2000; Fox *et al.*, 2002; Court *et al.*, 2003; Nomura *et al.*, 2004; Cai *et al.*, 2005; Takaishi *et al.*, 2009; Schmitz *et al.*, 2011). Progression of *H. felis*-stimulated carcinogenesis is accelerated in the hypergastrinaemic INS-GAS transgenic mouse model. INS-GAS mice develop spontaneous adenocarcinoma by 20 months of age; when infected with *H. felis* mice develop adenocarcinoma as early as 8 months of age (Wang *et al.*, 2000). This provides a convenient model to investigate host-bacterial interactions in *Helicobacter* pathogenesis over a shorter time frame.

1.6 The urokinase plasminogen activator system

1.6.1 Physiological and pathophysiological roles of the uPA system

The uPA system is an important regulator of tissue remodelling in both physiological and pathophysiological processes. The system, consisting of uPA, uPAR and the inhibitors, PAI-1 and PAI-2, regulates extracellular proteolysis via plasmin activation and affects cell adhesion and signalling to influence wound healing, chronic inflammation, fibrosis and cancer (Blasi & Carmeliet, 2002; Castellino & Ploplis, 2005; Smith & Marshall, 2010; Deryugina & Quigley, 2012). In the stomach, expression of uPA and PAI-1 is elevated at ulcer sites, and, in addition to uPAR, is stimulated by *Helicobacter* infection (Wodziński *et al.*, 1993; Herszenyi *et al.*, 1997; Kitadai *et al.*, 2003; Kenny *et al.*, 2008; Keates *et al.*, 2008). Increased expression of uPA, uPAR and PAI-1 is associated with increased invasiveness of gastric cancer cells and enhanced angiogenesis, correlating with adverse outcomes in gastric cancer (Nekarda *et al.*, 1994; Heiss *et al.*, 1995; Ganesh *et al.*, 1996; Lee *et al.*, 2003; Kaneko *et al.*, 2003; Kim *et al.*, 2005; Zhang *et al.*, 2006; Beyer *et al.*, 2006; Kim *et al.*, 2007; Kim *et al.*, 2008; Baek *et al.*, 2008; Kita *et al.*, 2009; Park *et al.*, 2011; Ding *et al.*, 2013).

1.6.2 The uPA system and extracellular proteolysis

uPA is a serine protease with a fundamental role in the plasmin-stimulated extracellular proteolysis, cleaving plasminogen at the single Arg^{561}-Val^{562} scissile bond to generate plasmin, a two-chain polypeptide complex linked by a single disulphide bond (Robbins *et al.*, 1967). The proteolytic activity of plasmin drives fibrinolysis during wound healing and also elicits ECM degradation via direct plasmin activity and activation of proteolytic MMPs such as MMP-2, MMP-3, MMP-9, MMP-12 and MMP-13 (Nagase *et al.*, 1990; Wong *et al.*, 1992; Carmeliet *et al.*, 1997; Monea *et al.*, 2002; Deryugina & Quigley, 2012). Extracellular proteolysis also liberates ECM-bound growth factors and activates latent growth factors and cytokines (Lyons *et al.*, 1990; Houck *et al.*, 1992; Campbell *et al.*, 1992; Whitelock *et al.*, 1996; Deryugina & Quigley, 2012). Plasmin-driven ECM degradation and growth factor activation facilitate cell migration and signalling, crucial for physiological and pathophysiological tissue remodelling processes including wound healing, angiogenesis, cancer cell proliferation, invasion and metastasis, as well as inflammatory cell recruitment (Castellino & Ploplis, 2005; Deryugina & Quigley, 2012). PAI-1, a member of the serine protease inhibitor family of proteins, has a regulatory role with regard to these processes, specifically inhibiting the plasminogen activators uPA and tissue plasminogen activator (tPA). PAI-1 binds covalently to uPA at its active site, and is itself inactivated by cleavage of the Arg-Met bond at its reactive centre (Andreasen *et al.*, 1986; Egelund *et al.*, 1998).

uPA is secreted as a single chain glycosylated pro-enzyme and is converted to the active enzyme, consisting of two polypeptide chains linked by a disulphide bond, by limited proteolysis (Skriver *et al.*, 1982; Neilsen *et al.*, 1982). Both uPA and pro-uPA bind with high affinity to uPAR, which is associated with the extracellular surface of the cell by a carboxyl-terminal glycosylphosphatidylinositol (GPI) anchor (Ploug *et al.*, 1991; Huai *et al.*, 2006). Binding of uPA and pro-uPA to uPAR localises proteolytic activity to the cell surface. Pro-uPA is reciprocally activated by plasmin generated by active uPA, amplifying proteolytic activity at the cell surface (Skriver *et al.*, 1982; Neilsen *et al.*, 1982). Directional migration of cells through the ECM is facilitated by localised expression of uPAR at the leading edge of migrating cells, concentrating proteolytic activity and ECM remodelling in the direction of migration (Estreicher *et al.*, 1990).

1.6.3 Cell adhesion, migration and signalling via uPAR

The uPA system mediates cell-ECM associations, via interactions between uPAR, integrins and the ECM component vitronectin (Wei *et al.*, 1994; Wei *et al.*, 1996). These interactions modulate cell adhesion, migration and intracellular signalling. Vitronectin is an ECM glycoprotein involved in cellular adhesion via interactions with integrins and is also an important element of cell migration, forming a matrix which the cell co-ordinately attaches to and detaches from as it migrates (Felding-Habermann & Cheresh, 1993; Waltz *et al.*, 1997). Integrins are transmembrane receptors that interact with various ECM and cell surface components to mediate cell adhesion and transduce signals arising from cell-ECM and cell-cell associations (Wei *et al.*, 1996). uPAR is also a high affinity receptor for vitronectin and forms stable complexes with integrins and caveolin (Wei *et al.*, 1994; Wei *et al.*, 1996; Wei *et al.*, 1999).

The stable complexes formed by the interactions between uPAR, $\alpha3\beta1$-integrin and caveolin alter the overall adhesive properties of $\beta1$-integrins, promoting adhesion via vitronectin (Wei *et al.*, 1996; Wei *et al.*, 1999; Wei *et al.*, 2001). Furthermore, interactions between uPAR and $\alpha5\beta1$-integrin enhance binding between this integrin isoform and the ECM component fibronectin (Wei *et al.*, 2005). uPAR can bind uPA and vitronectin simultaneously such that uPA binding uPAR enhances adhesion via vitronectin, promoting interactions between uPAR and integrins and increasing the affinity of uPAR-vitronectin binding (Wei *et al.*, 1994; Chapman & Wei, 2001; Sidenius *et al.*, 2002; Kjoller, 2002; Huai *et al.*, 2008).

uPAR-mediated cell adhesion and migration via vitronectin is inhibited by PAI-1, through both uPA dependent and independent pathways. Binding of PAI-1 to uPAR bound uPA results in inactivation of cell surface α_V-integrins and internalisation of the entire complex, along with integrins in complex with uPAR (Czekay *et al.*, 2003). The low-density lipoprotein receptor-related protein (LRP) mediates endocytic internalisation of this multi-protein complex; rapid clearance of uPAR and α_{V}-integrins in this way prevents reattachment of cells via vitronectin (Czekay *et al.*, 2003). PAI-1 also binds to vitronectin, stabilising PAI-1 in its active conformation in plasma and in the ECM (Wiman *et al.*, 1988; Seiffert *et al.*, 1991). The binding sites for PAI-1 and uPAR overlap in the amino terminal somatomedin B domain of vitronectin. Therefore

PAI-1 competitively inhibits uPAR binding (Deng *et al.*, 2001). Furthermore, PAI-1 in complex with vitronectin sterically hinders interactions via the RGD domain of vitronectin, inhibiting interactions between vitronectin and integrins (Stefansson *et al.*, 1996). PAI-1 can detach cells from the ECM by disrupting interactions between uPAR and integrins with vitronectin.

Signalling via GPI-anchored uPAR relies upon direct interactions of uPAR and/or vitronectin with transmembrane or internalisation receptors, to transduce the signal across the membrane into the intracellular compartment. These include integrins, GPCRs, receptor tyrosine kinases, such as EGFR, cytokine receptors, caveolin and endocytic receptor 180 (Blasi & Carmeliet, 2002; Smith & Marshall, 2010). Various intracellular signalling molecules and pathways can be activated via uPAR, including tyrosine kinases such as focal adhesion kinase (FAK) and Src, the mitogen-activated protein kinase (MAPK) pathway, the janus kinase/signal transducers and activators of transcription pathway, the phosphatidylinositol-3-kinase (PI3K)/Akt pathway and the Rho family GTPase Rac (Koshelnick *et al.*, 1997; Tang *et al.*, 1998; Nguyen *et al.*, 1998; Nguyen *et al.*, 2000; Kjoller & Hall, 2001; Blasi & Carmeliet, 2002; Liu *et al.*, 2002; Aguirre Ghiso, 2002; Vial *et al.*, 2003; Degryse *et al.*, 2005; Alfano *et al.*, 2006; Lester *et al.*, 2007; Smith & Marshall, 2010). The complex array of signalling pathways stimulated via uPAR modulates a variety of cellular functions, including survival, proliferation, differentiation, cytoskeletal properties, migration, adhesion and protease expression.

1.6.4 The uPA system in wound healing and fibrosis

The uPA system, together with tPA, critically regulates wound healing. Endothelial cell-derived tPA primarily drives plasminogen activation in the vasculature, stimulating fibrinolysis to prevent thrombosis (Andreasen *et al.*, 2000; Zorio *et al.*, 2008; Del Rosso *et al.*, 2011). Platelet-derived PAI-1 regulates fibrinolysis, binding to platelet-associated fibrin to stabilise the clot and prevent haemorrhage (Braaten *et al.*, 1993). A variety of parenchymal and stromal cell types express uPA, uPAR and PAI-1 during wound healing, to regulate the complex array of activities involved in tissue remodelling and acute inflammatory responses, via modulation of extracellular proteolysis, cell adhesion, migration and signalling (Blasi & Carmeliet, 2002; Smith & Marshall, 2010; Del Rosso *et al.*, 2011). Consequently, aberrant expression of the tPA/uPA system is implicated in pathological responses to tissue injury, including haemorrhage, thrombotic events, fibrosis and chronic inflammation.

The uPA system influences fibrosis, with tissue-specific effects (Zhang & Eddy, 2008). Fibrosis is a pathological tissue state characterised by excessive and disorganised deposition of ECM, often in response to tissue injury, which has deleterious effects on tissue structure and function (Knapp *et al.*, 1977; Hinz *et al.*, 2012). As described in section 1.2.1, persistent activation of myofibroblasts in the contracted wound results in deregulated ECM remodelling activity, leading to fibrosis (van den Borne *et al.*, 2010; Hinz *et al.*, 2012). Absence or deficiency of uPAR is associated with increased myofibroblast abundance and a fibrotic phenotype in kidney and skin (Zhang *et al.*, 2003a; Zhang *et al.*, 2003b; Kanno *et al.*, 2008). uPA attenuates lung and liver fibrosis, exacerbates cardiac fibrosis and has no effect upon kidney fibrosis (Salgado *et al.*, 2000; Hattori *et al.*, 2004; Moriwaki *et al.*, 2004; Heymans *et al.*, 2006; Yamaguchi *et al.*, 2007). PAI-1 is associated with an increase in myofibroblast abundance and is pro-fibrotic in various tissues (Oda *et al.*, 2001; Matsuo *et al.*, 2005; Tuan *et al.*, 2008; Hu *et al.*, 2009; Senoo *et al.*, 2010; Bauman *et al.*, 2010; Ghosh & Vaughan, 2012). A notable exception is cardiac tissue, where PAI-1 deficiency results in the development of spontaneous cardiac fibrosis in aged mice, although elevated PAI-1 is associated with injury-induced cardiac fibrosis (Moriwaki *et al.*, 2004; Ghosh *et al.*, 2010; Ghosh & Vaughan, 2012). Evidence from the above literature suggests that the uPA system can influence ECM turnover via effects on the recruitment, activation or persistence of myofibroblasts at the site of injury or by modulating plasmin/MMP-directed ECM degradation.

1.6.5 The uPA system in inflammation

The uPA system plays a significant role in inflammatory responses, mediating inflammatory cell activation and facilitating migration of leukocytes into the compromised tissue. uPAR is expressed by myeloid cells and activated T lymphocytes and is stimulated in response to various pro-inflammatory cytokines and bacterial products (Plesner *et al.*, 1994; Nykjaer *et al.*, 1994; Bianchi *et al.*, 1996; Del Rosso *et al.*, 2011). Similarly, uPA expression by various cell types, including epithelial, endothelial, monocytes, neutrophils and natural killer cells, is stimulated by pro-inflammatory cytokines, such as TNF-α, IL-1β, IL-2, IL-3 and bacterial products, such as lipopolysaccharide (LPS) (Mondino & Blasi, 2004; Del Rosso *et al.*, 2011). Injury, inflammation and infection elicit significant local and systemic elevations of PAI-1 (Quax *et al.*, 1990; Wang *et al.*, 2005; Samad *et al.*, 1996; Rijneveld *et al.*, 2003;

Renckens *et al.*, 2005; Renckens *et al.*, 2007; Kowal *et al.*, 2007; Kowal *et al.*, 2010; Budinger *et al.*, 2011; Lim *et al.*, 2011; Goolaerts *et al.*, 2011).

uPA and uPAR mediate various processes during inflammatory responses. uPA promotes neutrophil activation whilst uPAR facilitates neutrophil extravasation and migration (Abraham *et al.*, 2003; Gyetko *et al.*, 1995; May *et al.*, 1998). uPA stimulates the release of pro-inflammatory cytokines from monocytes and activates latent pro-inflammatory cytokines via plasminogen activation (Vaday & Lider, 2000; Del Rosso *et al.*, 2011). Furthermore, the extracellular proteolytic activity of uPA and uPAR facilitates migration of leukocytes into the site of inflammation, as illustrated by impaired macrophage migration in plasminogen deficient mice (Mondino & Blasi, 2004; Gong *et al.*, 2008).

Interactions between uPA and uPAR, in association with transmembrane co-receptors, are chemotactic to various inflammatory cells via proteolytic-independent mechanisms (Del Rosso *et al.*, 2011). For instance, uPAR-bound uPA is chemotactic to monocytes and basophils, predominantly via binding of a specific uPAR epitope to the GPCR FPRL1 and to FPRL2, respectively (Resnati *et al.*, 2002; de Paulis *et al.*, 2004). The chemotactic role of uPA-uPAR interactions during inflammation is highlighted by the fact that leukocyte recruitment is impaired in uPA$^{-/-}$ and uPAR$^{-/-}$ mice, leading to impaired host responses to bacterial infection and, ultimately, death (Gyetko *et al.*, 1996; Gyetko *et al.*, 2000; Gyetko *et al.*, 2001; Gyetko *et al.*, 2002; Rijneveld *et al.*, 2002).

1.6.6 The uPA system in cancer

Expression of uPA, uPAR and PAI-1 is elevated in various cancers, correlating with adverse outcomes and a poor prognosis (Kaneko *et al.*, 2003; Beyer *et al.*, 2006; He *et al.*, 2007; Dass *et al.*, 2008). Expression of uPA, uPAR and PAI-1 by cancer cells and stromal cells varies across different cancers. Expression of uPA, uPAR and PAI-1 is increased at the leading edge of invasive primary and secondary tumours of colorectal origin, primarily in the stromal compartment, where myofibroblasts express uPA and PAI-1; similarly myofibroblasts, and other stromal cells, express uPA, uPAR and PAI-1 in human mammary duct carcinoma microinvasive foci (Offersen *et al.*, 2003; Neilsen *et al.*, 2007; Illemann *et al.*, 2009). In oral squamous cell carcinoma, uPAR-expressing cells are predominantly stromal whilst the predominant PAI-1 expressing cell population is the neoplastic cells (Lindberg *et al.*, 2006). Strong expression of uPA by adenocarcinoma cells and expression of uPA, as well as strong expression of uPAR, by stromal myofibroblasts correlates with metastatic potential of pancreatic tumours (He *et al.*, 2007). In gastric cancer, cancer cells primarily express uPA and uPAR, with less expression by stromal cells including endothelial cells, whilst cancer cells and stromal cells express PAI-1 (Kaneko *et al.*, 2003).

Elevated expression of uPA and uPAR is associated with increased invasion and metastasis, via diverse pathways. The proteolytic activity of uPA-uPAR facilitates invasion of cancer cells through the basement membrane and ECM at the primary tumour site, intravasation, extravasation and invasion at metastatic foci (Mignati & Rifkin, 1993; Andreasen *et al.*, 2000; Dass *et al.*, 2008). Furthermore, non-proteolytic functions of the uPA system drive cancer cell migration, invasion and metastasis. uPAR promotes cell adhesion and migration on vitronectin, associated with morphological changes characteristic of a more motile phenotype. This is stimulated by direct interactions between uPAR and vitronectin and activation of Rac-1 (Kjoller & Hall, 2001; Masden *et al.*, 2007; Jo *et al.*, 2009; Pirazzoli *et al.*, 2013). Consequently, uPAR-expressing cells have increased metastatic potential in a mouse xenograft model (Jo *et al.*, 2009). Similarly, hypoxia stimulates uPAR expression in various cancer cell lines, inducing EMT. This is mediated by Rac-1 and PI3K/Akt activation and is associated with increased migration and invasion in a breast cancer cell line (Lester *et al.*, 2007). Breast cancer cell migration is also induced by uPAR binding uPA, stimulating FAK, cSrc and Shc phosphorylation and activation of the MAPK signalling pathway (Nguyen *et al.*, 1998; Nguyen *et al.*, 2000). Additionally, increased expression of the uPA system is implicated in endothelial cell migration and ECM remodelling required for tumour angiogenesis (Rakic *et al.*, 2003; Binder *et al.*, 2007).

uPA and uPAR influence cell proliferation, survival and differentiation via various pathways. uPA-uPAR acts through a complex of fibronectin, α5β1-integrin, EGFR and FAK to stimulate MAPK mediated proliferation of carcinoma cells, such that disruption of the interactions in this molecular complex or uPAR knockdown results in tumour cell dormancy *in vivo* (Yu et al. , 1997; Aguirre Ghiso, 1999; Aguirre Ghiso, 2002; Liu *et al.*, 2002). uPAR promotes survival via MAPK and PI3K/Akt mediated activation of Bcl-xL (Alfano *et al.*, 2006). uPA-uPAR stimulated MAPK activation promotes proliferation and suppresses apoptosis in several cancer cell lines (Hildenbrand *et al.*, 2008). Furthermore, uPAR induces cancer stem cell-like properties in breast cancer lines, increasing tumourigenicity of xenografted cells *in vivo* (Jo *et al.*, 2010).

The role of PAI-1 in cancer is somewhat complex and paradoxical. As an inhibitor of uPA-uPAR mediated proteolysis and signalling, one might reason that PAI-1 would protect against cancer progression. The results of some studies are in agreement with this hypothesis; PAI-1 transfected human prostate carcinoma cell xenografts display reduced tumourigenic, angiogenic and metastatic potential, whilst hyperthermia attenuates tumourigenesis and angiogenesis in murine mammary adenocarcinoma via an increase in PAI-1 expression (Soff *et al.*, 1995; Roca *et al.*, 2003). However, much evidence exists indicating that PAI-1 promotes tumourigenesis, angiogenesis, invasion and metastasis and is a poor prognostic indicator in a number of cancers, including gastric cancer (Nakamura *et al.*, 1992; Nekarda *et al.*, 1994; Heiss *et al.*, 1995; Herszenyi *et al.*, 1995; Ganesh *et al.*, 1996; Tsuchiya *et al.*, 1997; Plebani *et al.*, 1997; Bajou *et al.*, 1998; Swiercz *et al.*, 1998; Kawasaki *et al.*, 1998; Harbeck *et al.*, 2004; Leissner *et al.*, 2006; Leik *et al.*, 2006; Beyer *et al.*, 2006; Lin *et al.*, 2008; Nishioka *et al.*, 2012; Ding *et al.*, 2013). Evidence has emerged to suggest that PAI-1 elicits an inverted U-shaped dose-response curve in terms of angiogenesis, which could account for some of the conflicting observations reported on the role of PAI-1 in cancer progression (McMahon *et al.*, 2001; Devy *et al.*, 2002; Bajou *et al.*, 2004). uPA-independent pathways, involving interactions between PAI-1 and vitronectin that disrupt adhesion and signalling via uPAR, have the potential to modulate cell migration, proliferation, differentiation and apoptosis, and could account for the deleterious tissue remodelling activities of PAI-1. The role of PAI-1 in carcinogenesis is likely to be both tissue-dependent and dependent on the level of expression of PAI-1.

1.6.7 The uPA system in *Helicobacter* infection and gastric cancer

uPA, uPAR and PAI-1 are up-regulated in response to *H. pylori* infection in humans and in gastric cancer cell lines (Herszenyi *et* al, 1997; Iwamoto *et al.*, 2005; Kim *et al.*, 2005; Kim *et al.*, 2007; Iwamoto *et al.*, 2008; Keates *et al.*, 2008; Kenny *et al.*, 2008; Ikeda *et al.*, 2009). In gastric cancer cells, various mediators are implicated in the mechanisms increasing expression of uPA and uPAR, including the COX-2 / PGE2 pathway, and in the case of uPAR, reactive oxygen species-induced NF-κB signalling, MAPK, c-Jun N-terminal kinases (JNK) and activator protein 1 (AP-1) signalling pathways, increasing their invasiveness (Kim *et al.*, 2005; Kim *et al.*, 2007; Iwamoto *et al.*, 2008). Increased expression of uPA by *H. pylori* stimulates proliferation of gastric epithelial cells, via proteolytic release of HB-EGF (Kenny *et al.*, 2008). *H. pylori* increases PAI-1 expression in gastric cancer cells via the MAPK pathway (Keates *et al.*, 2008). Similarly, PAI-2 expression is increased in gastric cancer cells, via RhoA-dependent activation of NF-κB signalling and via paracrine interactions mediated by COX-2 products and IL-8, inhibiting apoptosis and invasion (Varro *et al.*, 2004).

As well as *H. pylori*, other agents stimulate uPA and uPAR expression in gastric cancer. uPA and uPAR expression is stimulated by macrophage inhibitory cytokine-1, via MAPK, and uPAR is additionally induced by EGF and macrophage-stimulating protein via MAPK, AP-1 and NF-κB signalling pathways, increasing gastric cancer cell invasiveness (Lee *et al.*, 2003; Baek *et al.*, 2008; Park *et al.*, 2011). Similarly, lysophosphatidic acid stimulates uPAR expression and gastric cancer cell invasiveness via Rho family GTPases, JNK, AP-1 and NF-κB signalling pathways (Kim *et al.*, 2008). *In vivo*, xenografted gastric cancer cells over-expressing uPA are highly tumourigenic and metastatic (Choi *et al.*, 2002).

1.7 Aims and Objectives

The primary objective of this thesis was to elucidate the role of the uPA system, particularly PAI-1, in regulating epithelial-mesenchymal interactions involved in maintaining gastric mucosal morphology, mucosal responses to chronic *Helicobacter* infection and responses to NSAID-induced gastric mucosal injury. The specific aims were to:

1. determine the role of PAI-1 in shaping gastric mucosal morphology, using both genetically modified mouse models and *in vitro* approaches;
2. determine the role of PAI-1 in influencing gastric myofibroblast gene expression and secretion of myofibroblast-derived proteins into the gastric mucosal microenvironment, and also examine the functional significance of these findings;
3. investigate the role of PAI-1 in determining gastric mucosal responses to chronic *Helicobacter* infection, using the *H. felis* mouse model;
4. investigate the role of PAI-1, uPA and uPAR in determining responses to NSAID administration, using an indomethacin-based model of gastric mucosal injury.

CHAPTER 2

MATERIALS AND METHODS

2.1 Materials

2.1.1 Chemicals and general reagents

All general use chemicals were sourced from Sigma (Poole, UK) or BDH Laboratory Supplies (Lutterworth, UK). Phosphate buffered saline (PBS; 10X, pH 7.4) was purchased from Gibco Life Technologies (Paisley UK) and was diluted 10-fold in deionised water prior to use. Formaldehyde solution (16%) was purchased from Agar Scientific (Stansted, UK) and was used as a 4% solution diluted in PBS. Bovine serum albumin (BSA) was purchased from Jackson ImmunoResearch Laboratories (Newmarket, UK) and was diluted in PBS, as stated. *In vivo* and *in vitro* proliferation assays were performed using 5-ethynyl-2'-deoxyuridine (EdU), provided as part of the Click-iT® EdU Alexa Fluor 488® Imaging kit (Invitrogen, Paisley, UK). The kit also provided dimethylsulfoxide (DMSO) to reconstitute the EdU stock solution, as well as EdU reaction buffer, buffer additive, $CuSO_4$, Alexa Fluor® 488 and Hoechst 33342 for EdU detection and nuclear counterstaining. Slides produced during immunohistochemistry, immunocytochemistry and EdU experiments were mounted using Vectashield or Vectashield with 4', 6-diamidino-2-phenylindole (DAPI) mounting medium (Vector Laboratories, Peterborough, UK).

2.1.2 Antibodies and antisera

Primary antibodies used for immunohistochemistry and immunocytochemistry are summarised in Table 2.1. Fluorescein isothiocyanate and Texas Red conjugated secondary antibodies (Jackson ImmunoResearch Laboratories) were raised in donkey and used at a dilution of 1:400. Donkey serum (Jackson ImmunoResearch Laboratories) was diluted to 10% in PBS for use as the blocking solution.

Primary Antibody	Host species	Immunogen	Source	Dilution
Monoclonal anti-α-SMA	Mouse	N-terminal acetylated N-terminal decapeptide of smooth muscle actin	Fitzgerald (Acton, MA, USA)	1:100
Polyclonal anti-desmin	Rabbit	Full length human desmin	Fitzgerald (Acton, MA, USA)	1:100
Polyclonal anti-dipeptidylpeptidase 4 (DPP4)	Chicken	Human DPP4 (amino acids 547-610)	Chemicon (Millipore, Watford, UK)	1:200
Anti-gastrin	Guinea Pig	Thyroglobulin conjugated C-terminal nonapeptide of human G17	Prof Andrea Varro, University of Liverpool	1:400
Polyclonal anti-H/K ATPase	Rabbit	GVRCCPGSWWDQ GLYY (C-terminal amino acids 1019-1034 of porcine H/K ATPase α-subunit)	Calbiochem (Merck Chemicals, Nottingham, UK)	1:200
Polyclonal anti-secretogranin-2	Rabbit	Ovalbumin conjugated peptide (N-terminal amino acids 1-19 of rat and human secretogranin-2)	Pierce Antibodies (Thermo Scientific, Rockford, IL, USA)	1:1000
Polyclonal Anti-vimentin	Guinea Pig	Purified vimentin from Calf lens	Fitzgerald (MA)	1:400

Table 2.1. Summary of primary antibodies used in immunohistochemistry and immunocytochemistry.

2.1.3 Primary cell isolation and culture

Cell culture plasticware was purchased from TPP (Trasadingen, Switzerland). Cell culture reagents were purchased from Sigma, with the exception of fetal bovine serum (FBS) and 0.25% Trypsin-ethylenediaminetetraacetic acid (-EDTA), which were purchased from Lonza (Slough, UK), and Collagenase A, which was purchased from Roche Diagnostics (Burgess Hill, UK).

2.1.4 Proteins, growth factors and drugs

Recombinant stabilised human PAI-1 was purchased from Calbiochem. This form of human PAI-1 has four amino acid substitutions that stabilise the molecule in its active conformation, even at elevated temperature and pH. Recombinant human EGF and recombinant human IGF-II were purchased from R&D Systems (Abingdon, UK). Indomethacin was purchased from Sigma.

2.2 Animals

2.2.1 Animal maintenance

C57BL/6 mice were purchased from Charles River Laboratories (MA, USA), unless stated otherwise, one-week prior to use in experimental procedures to allow for acclimatization. PAI-1 null (PAI-1$^{-/-}$) and uPAR null (uPAR$^{-/-}$) mice were originally purchased from JAX® MICE (Maine, USA) then bred in-house (Biomedical Services Unit, University of Liverpool) and were on a C57BL/6 background. PAI-1$^{-/-}$ and uPAR$^{-/-}$ mice are viable, fertile, and do not display any phenotypic abnormalities in the absence of challenge (Carmeliet *et al.*, 1993; Bugge *et al.*, 1995). PAI-1-H/Kβ and uPA-H/Kβ mice were also on a C57BL/6 background and were generated and bred as discussed in section 2.2.2. All mice were housed in a conventional, non-specific pathogen free animal facility, in a temperature, noise and humidity controlled environment with a strict 12h:12h light to dark light cycle, in polycarbonate-bottomed cages with environmental enrichment. Animals were fed a commercial pellet diet; food and water were provided *ad libitum*. Animals were humanely killed by exposure to a rising concentration of CO_2 followed by cardiac puncture, in accordance with Home Office Schedule 1 to the Animals (Scientific Procedures) Act 1986. Blood was collected in 0.109M tri-sodium citrate and plasma recovered by centrifugation (3000g, 15 min) then stored on ice or at -20°C prior to analysis. For experiments involving gastric cells or tissues, an abdominal incision was made down the linea alba to expose the viscera. All experiments involving animals were approved by the Animal Welfare Committee at The University of Liverpool, and were Home Office licensed under the Animals (Scientific Procedures) Act 1986. Unless otherwise stated, experiments were performed on mice aged 10-13 weeks.

2.2.2 Generation of PAI-1-H/Kβ, uPA-H/Kβ and PAI-1$^{-/-, TG+}$ mice

Mice with targeted expression of PAI-1 or uPA to parietal cells had been generated in this laboratory, in collaboration with Dr Nikolina Vlatkovic at the University of Liverpool (Kenny *et al.*, 2013a). The coding sequence of mouse PAI-1 or uPA cDNA had been inserted downstream of approximately 1.1 kilobase pairs of the promoter region of the mouse H/K ATPase β-subunit gene (-1082 - +24) to target gene expression specifically to parietal cells (Figure 2.1). The resulting founders of PAI-1-H/Kβ and uPA-H/Kβ mice were cross-bred into C57BL/6 mice and maintained in house, by breeding homozygous (transgene +/+) offspring. Targeted expression of the

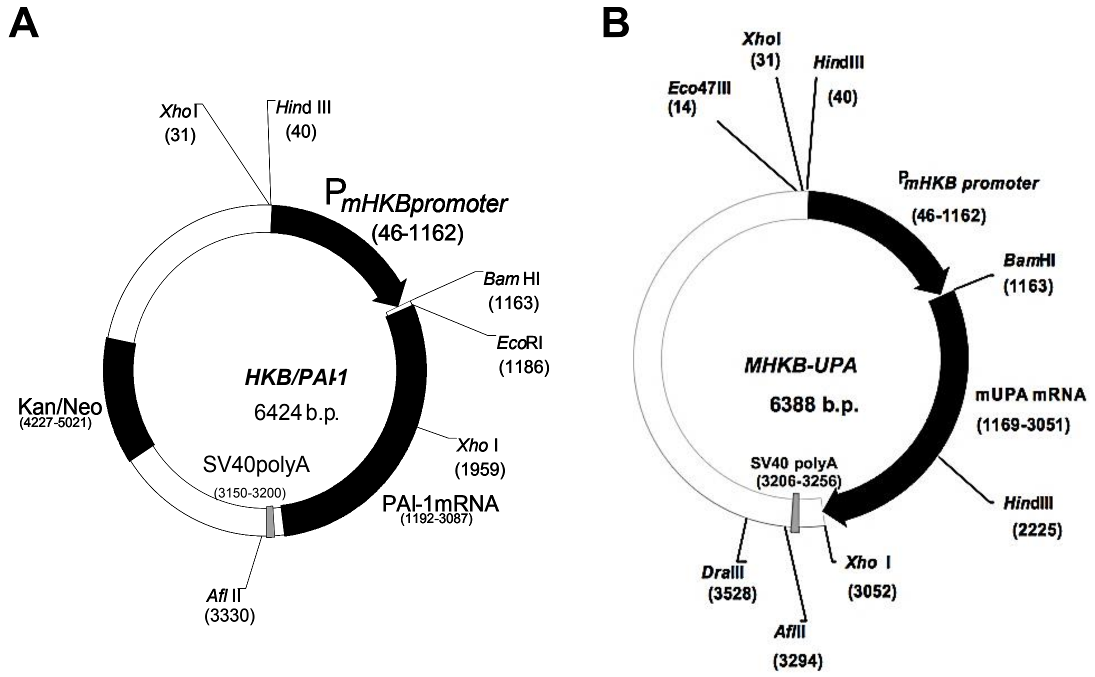


Figure 2.1. **Generation of mice with targeted expression of PAI-1 or uPA to parietal cells.** Representation of the transgene vector used to generate mice with targeted expression of **A:** PAI-1 and **B:** uPA to parietal cells. Restriction sites at Hind III (40) and Afl II (3330) were used to isolate the transgene from the vector.

PAI-1 transgene by PAI-1-H/Kβ mice had been confirmed by polymerase chain reaction, with positive expression by gastric corpus but not heart, liver, gastric antrum, spleen nor kidney. Mice that express only transgenic PAI-1 (PAI-1$^{-/-,\ TG+}$) were generated by crossing PAI-1$^{-/-}$ with PAI-1-H/Kβ mice, producing a dihybrid (wild-type and transgenic PAI-1) heterozygous F1 generation, from which mice with the desired genotype were produced in the F2 generation.

2.2.3 Murine *Helicobacter felis* infection

H. felis (ATCC 49179) was cultured by Ms Debbie Sales (Medical Microbiology, The University of Liverpool). Mice were infected with *H. felis* by Dr Susan Kenny. Animals aged 6-8 weeks were gavaged with 0.5ml of a *H. felis* suspension in TBS broth, three times over a 1-2 week period. In order to ensure quality control of the bacteria, the optical density of suspension was above 1.5 and *H. felis* had been passaged no more than 5 times. Positive *H. felis* infection was confirmed by rapid antral urease test (Prontodry, Medical Instruments Corporation, Solothrum, Switzerland) or by antral histology. After the mice had been infected for 46 weeks they were humanely killed together with age- and sex-matched uninfected animals of each strain. Tissue was taken for preparation of frozen sections or paraffin-embedded sections (section 2.3 and 2.4 respectively).

2.2.4 Indomethacin

In optimisation studies, animals were fed *ad libitum* or fasted for 18h prior to treatment, with access to water *ad libitum*. Animals were dosed with 100μl of either 20mg/kg indomethacin in 5% sodium bicarbonate or vehicle alone, by gavage or by intraperitoneal (IP) injection. Animals were humanely killed 6, 17 or 24h after treatment, blood taken, and the stomach removed, opened along the lesser curvature and washed in PBS. The stomach was then carefully stretched, mucosa side up, on filter paper and lesions scored. The length of each lesion was measured using a millimetre ruler. Total lesion length for each stomach was calculated as the sum of the lengths of each individual lesion. Lesion scores were more consistent using the oral route of administration compared to IP injection, and were both higher and more consistent 6h after indomethacin compared to 17h. No lesions were present 24h after indomethacin. Similarly there were no apparent lesions on stomachs taken from mice that had been fed *ad libitum.* Therefore in all subsequent studies, indomethacin was administered by gavage and animals were killed 6 hours after indomethacin. Following lesion scoring and imaging, stomachs were prepared for frozen tissue sections (section 2.3).

2.3 Preparation of frozen gastric tissue sections

The isolated stomach was opened along the lesser curvature, washed in PBS, carefully stretched mucosa side up and pinned on wax, before being fixed at room temperature in 4% formaldehyde for 1h. The stomach was then washed in PBS, placed in 20% sucrose in PBS overnight at 4°C, washed again in PBS and a strip of corpus and/or antrum tissue cut from just below the non-glandular region (for corpus) or just below the corpus region (for antrum) to a distance of ~2-3mm distal to this point (Figure 2.2A). Strips of tissue were placed upright on a cork disc supported between pins, with the most distal part of the section in contact with the cork (Figure 2.2B). The tissue was fixed onto the cork in embedding compound (CRYO-M-BED, Bright Instrument, Huntingdon, UK) by rapid freezing with Cryospray 134 (Bright Instrument). The pins were removed and the frozen tissue stored at -80°C. Cryosections of 7µm thickness were cut and captured on polylysine coated slides. The position of the tissue on the cork was such that serial sectioning through the tissue, parallel to the cork surface, produced full thickness sections of the gastric mucosal and submucosal layers (Figure 2.2B). Sections were stored at -80°C.

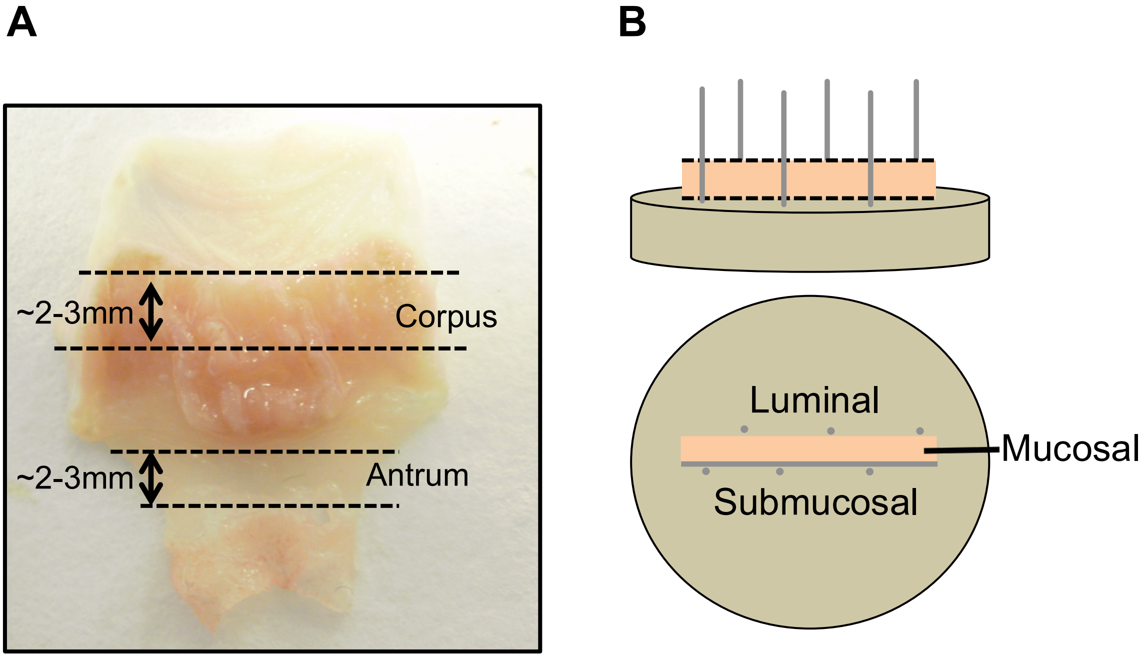


Figure 2.2. Arrangement of corpus and antrum tissue taken for cryosectioning. A: Strips of corpus and antrum tissue were cut, at a depth of ~2-3mm from proximal to distal cutting loci. **B:** The tissue strip was placed onto a cork disc between pins for freeze-fixing, in such an orientation that cryosectioning produced full thickness sections through the mucosal and submucosal layers.

2.4 Paraffin embedded tissues

Tissues for paraffin embedding were prepared by Dr Susan Kenny. The exposed stomach was ligated at the oesophageal and pyloric sphincters, carefully inflated with 4% formalin (Sigma) in PBS and placed in 4% formalin in PBS for 24h at room temperature. A ring of tissue was then cut from corpus and/or antrum, at the same locations as for frozen tissue strips. The ring of tissue was placed into a tissue processing cassette in 70% ethanol and processed for serial sectioning. These were then stained with haematoxylin and eosin (H&E) and, in the case of tissues from *H. felis* experiments, also with Giemsa stain or were immunostained for vimentin. Paraffin embedding, sectioning and staining were carried out by The University of Liverpool Veterinary Laboratory Services (Histology).

2.5 Mouse gastric gland isolation and culture

The procedure used to isolate gastric glands was based on a recently modified method, first described by Berglindh & Obrink (Berglindh & Obrink, 1976; Pagliocca *et al.*, 2008). The exposed stomach was ligated at the oesophageal and pyloric sphincters and excised by cutting beyond the ligation points. Adherent tissue was removed and the stomach washed in ice-cold Hanks' Balanced Salt Solution (HBSS) with 25mM $NaHCO_3$ (used throughout). A small section of non-glandular stomach was removed at the tip and the stomach fully inverted by guiding the ligated pyloric sphincter carefully through the gap. The opening in the non-glandular stomach was ligated, just beyond the junction with the corpus region, so that the entire glandular region formed a fully-sealed pouch. Collagenase A (0.5mg/ml, 1ml) in HBSS was carefully injected into the stomach and the inflated stomach put into ice cold HBSS. Stomachs were washed in 5ml 37^{o}C HBSS 3 times then incubated in 5ml 1mM dithiothreitol (DTT) in HBSS for 15 min at 37^{o}C, continuously gassing with 5% CO_2 / 95% air and shaking at 100 cpm. Stomachs were then washed again 3 times in 37^{o}C HBSS and similarly incubated in 0.4mg/ml collagenase A in HBSS until they deflated i.e. digestion through the wall (~ 5 – 30 min). Stomachs were then triturated with pressure from a wide-mouthed pipette ~10 times. The suspension was decanted and large tissue particles allowed to sediment for 45 seconds. The supernatant was then carefully transferred to a new vessel, shaken vigorously to release glands then re-sedimented for 45 min, after which the supernatant was carefully pipetted and discarded, leaving the sedimented glands in a small volume. Glands from each animal were equally divided into 4 wells of a 24-well plate and cultured on cover slips in ~2ml Dulbecco's Modified Eagle's Medium (DMEM)/Hams F12 supplemented with 10% FBS, 1% L-glutamine and 1% antibiotic antimycotic solution, in a sterile humidified chamber at 37°C in 5% CO_2 / 95% air for 48h, changing the medium after 24h. Glands were then cultured in 1ml / well DMEM/Hams F12 supplemented with 1% L-glutamine and 1% antibiotic-antimycotic solution (serum-free medium) for 24h, prior to treatment.

2.6 Mouse gastric myofibroblast isolation and culture

The procedure used to isolate gastric myofibroblasts was based on a method developed by Wu *et al.* (Wu *et al.*, 1999). The stomach was rapidly removed and washed in ice cold HBSS without Ca^{2+} and Mg^{2+} (used throughout). The antrum was isolated and placed in ice cold HBSS. The tissue was then cut into pieces of approximately $2.5mm^2$ and incubated in 1mM DTT in HBSS for 15 min at room temperature with continuous gentle shaking. The tissue pieces were then washed twice in ice cold HBSS and incubated in 0.02% EDTA for 30 min at 4^oC with continuous gentle shaking. This process of washing in HBSS followed by incubation in EDTA was repeated ~7-10 times. Following denudation of epithelial cells, antral pieces from each animal were cultured in a $T25cm^2$ flask in Roswell Park Memorial Institute (RMPI)-1640 medium supplemented with 10% FBS, 2% antibiotic antimycotic solution and 1% penicillin-streptomycin solution (immature myofibroblast medium), in a sterile humidified chamber at 37°C in 5% CO_2 / 95% air. The culture medium was changed every 24h for the first 72h, then every 48-72h, until myofibroblasts were plated to the bottom of the flask at ~50% confluence. Tissue pieces were then carefully removed and myofibroblasts cultured in DMEM supplemented with 10% FBS, 2% antibiotic antimycotic solution, 1% penicillin-streptomycin solution and 1% non-essential amino acids (mature myofibroblast medium). Culture medium was changed every 48-72h and cells were routinely passaged at confluence by incubation with 0.25% trypsin-EDTA for ~7 min, or as soon as cells were fully detached from the flask bottom.

2.7 Human myofibroblasts

Myofibroblasts were isolated from the resected antrum of a transplant donor by Dr Peter Hegyi, Department of Medicine, University of Szeged, Hungary, with the approval of the Ethics Committee of the University of Szeged. The protocol used to isolate and culture human myofibroblasts was similar to that used to isolate mouse myofibroblasts, the only difference being that the antral mucosal layer was dissected from the submucosal and muscle layers and cut into ~1cm^2 pieces prior to incubation in DTT. Myofibroblasts were isolated and cultured as described in section 2.6.

2.8 PAI-1 enzyme-linked immunosorbent assay

The concentration of PAI-1 in mouse plasma was determined by enzyme-linked immunosorbent assay (ELISA) (Murine PAI-1 total antigen assay, Molecular Innovations, Novi, MI, USA). Aliquots of neat plasma were plated in duplicate and the assay performed according to the manufacturers instructions. Absorbance at 450nm was detected using a SpectraCount plate reader (Packard Instruments, Meriden, CT, USA) and values were corrected by subtracting the mean absorbance of the zero standards from all readings. The concentration of PAI-1 in plasma samples was read from a standard curve of [PAI-1].

2.9 Immunohistochemistry

Frozen gastric sections (section 2.3) were thawed in deionised water for 30 seconds and dehydrated in 50% ethanol for 10 min followed by 75% ethanol for 10 min. Sections were rinsed in deionised water, blocked with 10% donkey serum for 30 min and then covered with primary antibody, diluted in 1% BSA in PBS (Table 2.1), and incubated overnight at 4^{o}C in a humidified chamber. Negative controls were generated by incubating sections with 1% BSA in PBS without primary antibody. The following day sections were washed for 5 min in the following series of NaCl solutions: 0.14M, 0.5M and 0.14M. Sections were then covered with secondary antibody diluted 1:400 in 10mM 4-(2-hydroxyethyl)-1-piperazineethanesulphonic acid (HEPES) pH7.6, and incubated for 1h in the dark. Sections were protected from the light for the remainder of the protocol. Secondary antibody was blotted off and sections were washed in 3 changes of PBS, 10 min per wash. Sections were mounted with Vectashield containing DAPI and slides were stored in the dark at 4^{o}C.

2.10 Immunocytochemistry

Media was removed from cells cultured on cover slips and cells were washed twice with PBS then fixed in 4% formaldehyde in PBS for 30 min. Cells were washed twice with PBS then permeabilised in PBS with 0.3% BSA and 0.2% Triton X-100 for 30 min, blocked with 5% BSA for 30 min and 10% donkey serum for 30 min, with two washes in PBS between each incubation. Cells were then incubated overnight with primary antibody, diluted in PBS (Table 2.1), at 4^{o}C in a humidified chamber. Negative controls were generated by simultaneously incubating some cells with PBS alone. The following day, primary antibody was removed and cells were washed for 10 min in the following series of NaCl solutions: 0.14M, 0.5M and 0.14M. Cells were then incubated with secondary antibody, diluted 1:400 in 10mM HEPES pH7.6, for 1h in the dark. Cells were protected from the light for the remainder of the protocol. Secondary antibody was removed and cells were washed in 3 changes of PBS, 10 min per wash. Cover slips were mounted on slides with Vectashield containing DAPI and slides were stored in the dark at 4^{o}C.

2.11 EdU incorporation and detection

EdU incorporation and detection was carried out according to the protocol provided with the Click-iT® EdU Alexa Fluor® 488 imaging kit. A 10mM EdU stock solution was prepared by reconstitution in DMSO. EdU solution was added to cells cultured on cover slips at a final concentration of 10µM in culture medium and allowed to incubate for 2h in a sterile humidified chamber at 37°C in 5% CO_2 / 95% air. The medium was then removed and cells washed twice in PBS followed by fixation in 4% formaldehyde in PBS for 30 min at room temperature. This was followed by 2 washes with 3% BSA in PBS and 2 washes with PBS. Cells were permeabilised with 0.5% Triton X-100 in PBS for 20 min at room temperature. The EdU reaction cocktail was prepared by combining 1X Click-iT® reaction buffer, $CuSO_4$, Alexa Fluor 488® and 1X reaction buffer additive, as specified in the manufacturer's protocol. Following permeabilisation, cells were washed twice with 3% BSA in PBS then incubated with EdU reaction cocktail for 30 min at room temperature, in the dark. Cells were protected from light throughout the remainder of the protocol. Cells were then washed once with 3% BSA in PBS then twice with PBS. If immunocytochemistry was being performed on the cells, the remainder of the protocol followed the description in section 2.10, from the donkey serum blocking step onwards. Otherwise nuclei were counterstained with 2µg/ml Hoechst 33342 for 30 min at room temperature. Cells were then washed twice with PBS and the cover slips were mounted on slides with Vectashield. Slides were stored in the dark at 4^{o}C.

2.12 Microscopy and imaging

Slides were examined under a Zeiss Axioplan-2 microscope (Carl Zeiss, Cambridge, UK) connected to an Axiocam HRm camera (Carl Zeiss) for imaging. Microscopy and imaging were performed at 10X or 40X magnification (air immersion) or 63X magnification (oil immersion). Images were processed with AxioVision 4.5 software (Carl Zeiss). The identities of slides were masked and slides assigned an arbitrary identification number until all samples had been assessed. Reference slides were examined at the commencement of and during sessions evaluating graded parameters, to reduce intraobserver variability.

The mucosal thickness of DAPI or H&E stained corpus and/or antrum sections was determined by imaging at 10X magnification and use of the length measurement tool available with Axiovision 4.5. (Figure 2.3). Myofibroblast abundance and parietal cell counts were determined under 40X magnification. Myofibroblast abundance was evaluated using a grading system of 0 – 4 for vimentin immunostaining (Figure 2.4). Parietal cells were quantified as the number of H/K ATPase immunopositive cells along full mucosal thickness in the width of the visual field (600μm), and expressed as number of parietal cells / 100μm. For all measurements, 5 equidistant fields per section were examined, in at least 2 well orientated sections, and the mean or median calculated.

For histopathological scores, H&E stained sections were examined under 10X and 40X magnification. Inflammation, epithelial defects, foveolar hyperplasia and oxyntic gland atrophy were quantified using a previously developed scale (Rogers *et al.*, 2005). Inflammation was scored as follows: Grade 0 = no inflammation; Grade 1 = patchy leukocyte infiltration into mucosa / submucosa; Grade 2 = multifocal or coalescing mucosal / submucosal leukocyte infiltration; Grade 3 = lymphoid follicles, extension into tunica muscularis; Grade 4 = effacing transmural inflammation. Epithelial defects were scored as follows: Grade 0 = no defects; Grade 1 = rare dilated glands, Grade 2 = frequent dilated glands, surface epithelial tattering; Grade 3 = glandular atrophy, surface erosions; Grade 4 = glandular atrophy with ulceration and fibrosis. Foveolar hyperplasia scores were defined as: Grade 0 = normal isthmus length, Grade 1 = ~1.5x, Grade 2 = ~2x, Grade 3 = ~3x, Grade 4 ≥ 4x normal isthmus length. Atrophy was quantified as: Grade 0 = no parietal of chief cell loss; Grade 1 = ~ 25% parietal cell loss, ~ 50% chief cell loss; Grade 2 = ~ 50 % parietal cell loss, complete chief cell loss; Grade 3 = ~ 75% parietal cell loss, complete chief cell loss; Grade 4 = > 75% parietal cell loss, complete chief cell loss. Giemsa staining was examined under 63X magnification, and graded from 0 – 4 according to the frequency of colonised glands and density of colonisation.

2.13 Microarrays

Mouse antral myofibroblasts were isolated, cultured and RNA extracted by Dr Isaly Steele (section 2.6). Myofibroblasts used for microarrays were passaged no more than 4 times. These myofibroblasts were characterised on the basis of α-SMA and vimentin expression by immunocytochemistry and were determined to be homogeneous cultures, with >99% of cells expressing these markers of myofibroblast differentiation. RNA was extracted using the RNeasy Mini Kit (Qiagen, Crawley, UK), according to the manufacturer’s instructions. Reverse transcription, labelling, fragmentation and hybridisation of cDNA to the GeneChip® Mouse Genome 430 2.0 array (Affymetrix, High Wycombe, UK) and scanning of the

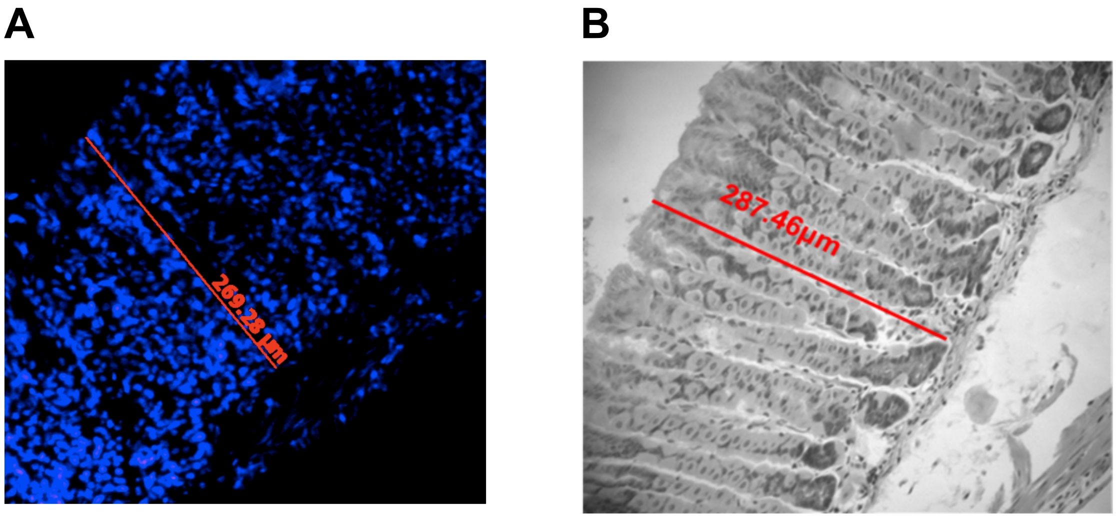


Figure 2.3 Measuring mucosal thickness. A. DAPI filter for imaging cellular nuclei of frozen corpus sections. **B.** H&E stained section. In both cases, mucosal thickness was determined as the length from the muscularis mucosae to the surface of the mucosa.

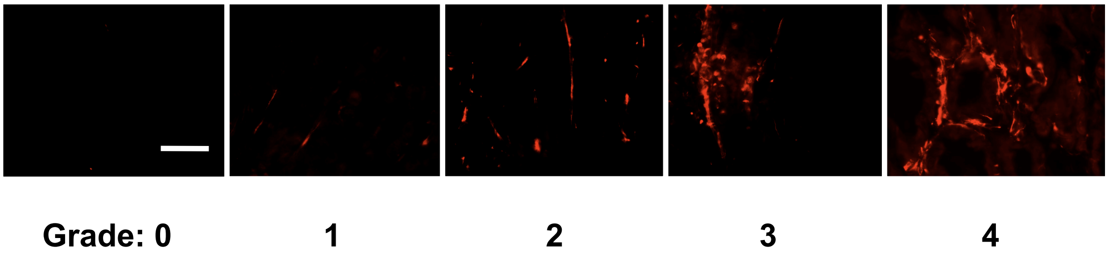


Figure 2.4. Myofibroblast abundance. Representative images of each grade used to quantify expression of vimentin, a myofibroblast marker. Grade 0: no staining in field; grade 1: rare staining (1-2 myofibroblasts per gland, with long spindle-shaped morphology); grade 2: mild / moderate staining (multiple long spindle-shaped myofibroblasts per gland); grade 3: extensive staining (many myofibroblasts in field, some branching morphology); grade 4: heavy staining throughout field (branching aggregates of myofibroblasts throughout field). Scale bar = 50μm.

array, using the GeneChip® Scanner 3000 (Affymetrix), was carried out by Dr Lucille Rainbow at the Liverpool Genome Facility, according to the protocol provided by Affymetrix. Analysis of raw signal intensity values generated by the microarray was accomplished using GeneSpring GX 10 (Agilent Technologies, Wokingham, UK). Affymetrix Microarray Suite 5.0 (MAS5.0) summary algorithm was used to normalise the data and probe sets were assigned a flag of present (P), marginal (M) or absent (A), based on normalised signal intensities. Data were compared as two groups: wild-type (C57BL/6) vs. PAI-$1^{-/-}$ myofibroblasts. Only probe sets that were flagged as present in all samples of at least one group were included for further analyses through MetaRodent™ pathway analysis software (GeneGo, St Joseph, MI, USA).

2.14 DPP4 activity assays

DPP4 activity of cultured human myofibroblasts was determined using a colorimetric assay based on the generation of the chromogenic molecule p-nitroaniline (pNA) via cleavage of Gly-Pro-pNA by DPP4 (Nagatsu *et al.*, 1976) A solution of Tris-buffered saline (TBS; 30mM Tris, 150mM NaCl, pH 8.0) was prepared as the diluent for these experiments. A stock solution of 1mM 4-nitroaniline (pNA; Sigma) was diluted in TBS to produce standards. The standards were set at 0, 20, 40, 80 and 160nM pNA. The substrate for these experiments was 3mM gly-pro-pNA (Sigma). Following plating and treatment of cells, the assay was set-up, with standards, blanks and experimental conditions plated in duplicate. Standards were plated at 200µl / well. 200µl / well TBS was added to cells, following removal of culture medium and 3 washes with TBS. An additional 100µl TBS was added to cells in the blank wells and to the standards and 100µl substrate was added to all other cells. The plate was incubated at 37°C for 30 min, then absorbance at 405nm was detected using a

SpectraCount plate reader (Packard Instruments, Meriden, CT, USA). Absorbance values were corrected by subtracting the mean absorbance of the blank wells from all readings. Generation of pNA, equating to DPP4 activity, was read from a standard curve of [pNA].

A similar strategy was used to determine DPP4 activity of mouse plasma, using a fluorimetric assay based on the generation of the fluorophore 7-amino-4-methylcoumarin (AMC) via cleavage of the substrate Gly-Pro-7-amido-4-methylcoumarin hydrobromide (Gly-Pro-AMC; Sigma) by DPP4 (Kato *et al.*, 1978). Standards, blanks and experimental conditions were plated in duplicate and fluorescence values were averaged. Standards were set at 0, 2, 5, 10 and 20nmol AMC, taken from a 1mM stock solution (Sigma) and made up to 40µl / well with TBS. Aliquots of neat plasma were added to wells at the required volume and made up to 40µl with TBS. An additional 40µl TBS was added to blank wells and standards and 40µl 0.1mM Gly-Pro-AMC was added to all other wells. The plate was incubated for 20 min at room temperature then fluorescence at 360nm excitation / 460nm emission was detected using a FluoroCount plate reader (Packard Instruments, Meriden, CT, USA). Values were corrected by subtracting blanks, and generation of AMC, equating to DPP4 activity, was read from a standard curve of AMC (nmol).

2.15 Statistics

Group data are presented as mean ± standard error of the mean (SEM), or median ± range / interquartile range (IQR) for categorical scores and grades. Comparisons between experimental groups were made using an unpaired Student's *t*-test or one-way analysis of variance (ANOVA) with Tukey's post hoc analysis for multiple comparisons, for parametric data. Comparisons between groups of non-parametric data were made using a Mann-Whitney *U*-test or Kruskal-Wallis ANOVA on ranks test with Dunn's post hoc analysis for multiple comparisons. Differences between groups were considered significant at $P < 0.05$.

CHAPTER 3

THE ROLE OF PAI-1 IN DETERMINING GASTRIC MUCOSAL MORPHOLOGY

3.1 Introduction

Gastric PAI-1, expressed primarily by parietal and ECL cells, is elevated in response to *H. pylori* infection and hypergastrinaemia, both of which alter gastric mucosal morphology (Kenny *et al.*, 2008; Keates *et al.*, 2008; Norsett *et al.*, 2011). PAI-1 may modulate the tissue remodelling activity of the uPA system, influencing proliferation, differentiation, migration and survival of parenchymal and mesenchymal cell populations, via uPA-dependent antiproteolytic activity and by disrupting signalling via uPAR, as described in sections 1.6.2 and 1.6.3 respectively. Previous work in this laboratory has demonstrated that PAI-1 attenuates *H. pylori*-induced gastric epithelial cell proliferation, by inhibiting uPA-mediated proteolytic release of HB-EGF (Kenny *et al.*, 2008). Therefore, the level of PAI-1 expression in the gastric mucosa is likely to shape mucosal morphology.

Mice have been generated in this laboratory with targeted expression of PAI-1 to parietal cells, driven by the H/K ATPase β-subunit promoter, as described in section 2.2.2. These mice (PAI-1-H/Kβ), have significantly increased gastric expression of PAI-1 mRNA, elevated concentrations of circulating PAI-1, are moderately obese, hyperphagic and are resistant to CCK-evoked satiety signals (Kenny *et al.*, 2013a). The work in this chapter explores the gastric mucosal phenotype of PAI-1-H/Kβ mice, in comparison to commercially available PAI-1$^{-/-}$ and wild-type mice.

3.1.1 Aims and objectives

The aim of this chapter was to determine the role of PAI-1 in influencing gastric corpus mucosal morphology. The specific objectives were to:

1. determine the concentration of PAI-1 in plasma from C57BL/6, PAI-1$^{-/-}$ and PAI-1-H/Kβ mice;
2. compare gastric corpus mucosal thickness, parietal cell abundance and myofibroblast abundance of C57BL/6, PAI-1$^{-/-}$ and PAI-1-H/Kβ mice;
3. determine the effect of endogenous and exogenous PAI-1 on basal and growth factor-stimulated gastric epithelial cell proliferation.

3.2 Methods

3.2.1 PAI-1 ELISA

Plasma from C57BL/6, PAI-1$^{-/-}$ and PAI-1-H/Kβ mice aged 9-12 weeks was prepared as described in section 2.2.1. Samples were plated in duplicate for the PAI-1 ELISA, which was performed as described in section 2.8.

3.2.2 Histology

Stomachs were processed for either frozen sections or paraffin-embedded sections, as described in sections 2.3 and 2.4. Paraffin-embedded sections were stained with H&E. Frozen sections were prepared for immunofluorescence.

3.2.3 Histological analysis

Corpus mucosal thickness of C57BL/6, PAI-1$^{-/-}$ and PAI-1-H/Kβ mice was determined by measuring the distance from the muscularis mucosae to the luminal surface on images of H&E or DAPI stained sections, as described in section 2.12. Corpus myofibroblasts were identified using vimentin immunofluorescence and their abundance was graded from 0 – 4, as described in section 2.12. Parietal cells were identified using H/K ATPase immunofluorescence and their abundance was quantified as described in section 2.12.

3.2.4 *In vitro* EdU proliferation assay

Gastric glands were isolated and cultured as described in section 2.5. Cultured glands were incubated in serum-free medium for 24 hours prior to treatment with

PAI-1, EGF or IGF-II and finally were incubated with 10μM EdU for 2 h, fixed and prepared for EdU detection, as described in section 2.11. Epithelial cell proliferation in each gland was calculated as the percentage of cells that were EdU positive; 10 glands per cover slip were evaluated.

3.2.5 Statistics

Data points for mucosal thickness and parietal cell numbers represent mean values for individual animals, whilst data points for vimentin grades represent the median value. Group data are presented as mean ± SEM, or median ± IQR for vimentin grades. Comparisons between groups were made using a one-way ANOVA with with Tukey's post hoc analysis for multiple comparisons, or Kruskal-Wallis ANOVA on ranks test with Dunn's post hoc analysis for multiple comparisons for vimentin grades, and were considered significant at $P < 0.05$.

3.3 Results

3.3.1 PAI-1-H/Kβ mice have elevated concentrations of circulating PAI-1

The mean concentration of PAI-1 in plasma of PAI-1-H/Kβ was ~3-fold higher than wild-type plasma (Figure 3.1). There was no detectable PAI-1 in the plasma of PAI-1$^{-/-}$ mice.

3.3.2 PAI-1-H/Kβ mice have increased corpus mucosal thickness

The gastric phenotype of male and female mice was compared, and no obvious differences were found. Consequently, here and in the following sections, data from male and female mice have been combined to produce the group data. Mean corpus mucosal thickness of 53-56 week old PAI-1-H/Kβ mice was ~2-fold higher than C57BL/6 and PAI-1$^{-/-}$ mice (Figure 3.2A). Both gland and pit lengths were increased in PAI-1-H/Kβ mice, accounting for the increased mucosal thickness (Figure 3.2B). Gross mucosal morphology of PAI-1$^{-/-}$ and PAI-1-H/Kβ corpus tissues was otherwise similar to wild-type (Figure 3.2A).

3.3.3 Abundance of corpus mucosal myofibroblasts and parietal cells is similar in C57BL/6, PAI-1$^{-/-}$ and PAI-1-H/Kβ mice

The median vimentin grade of corpus mucosa from wild-type, PAI-1$^{-/-}$ and PAI-1-H/Kβ was similar (Figure 3.3A), representing 1-2 myofibroblasts within the lamina propria separating each gland (Figure 3.3B). Myofibroblasts were equally distributed along the entire length of the lamina propria in all three strains, aligning with sections of glands and pits from base to lumen with similar frequency.

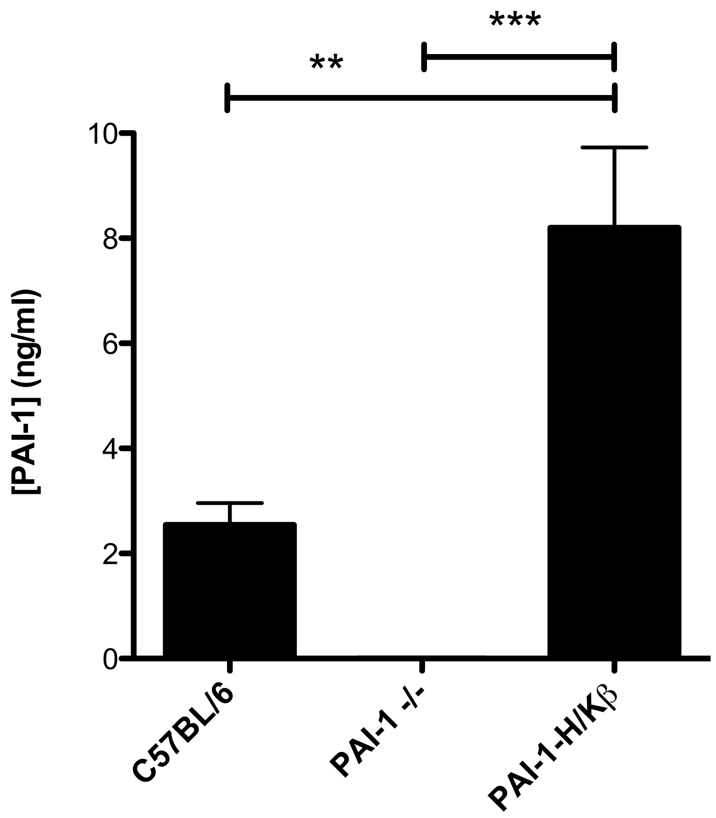
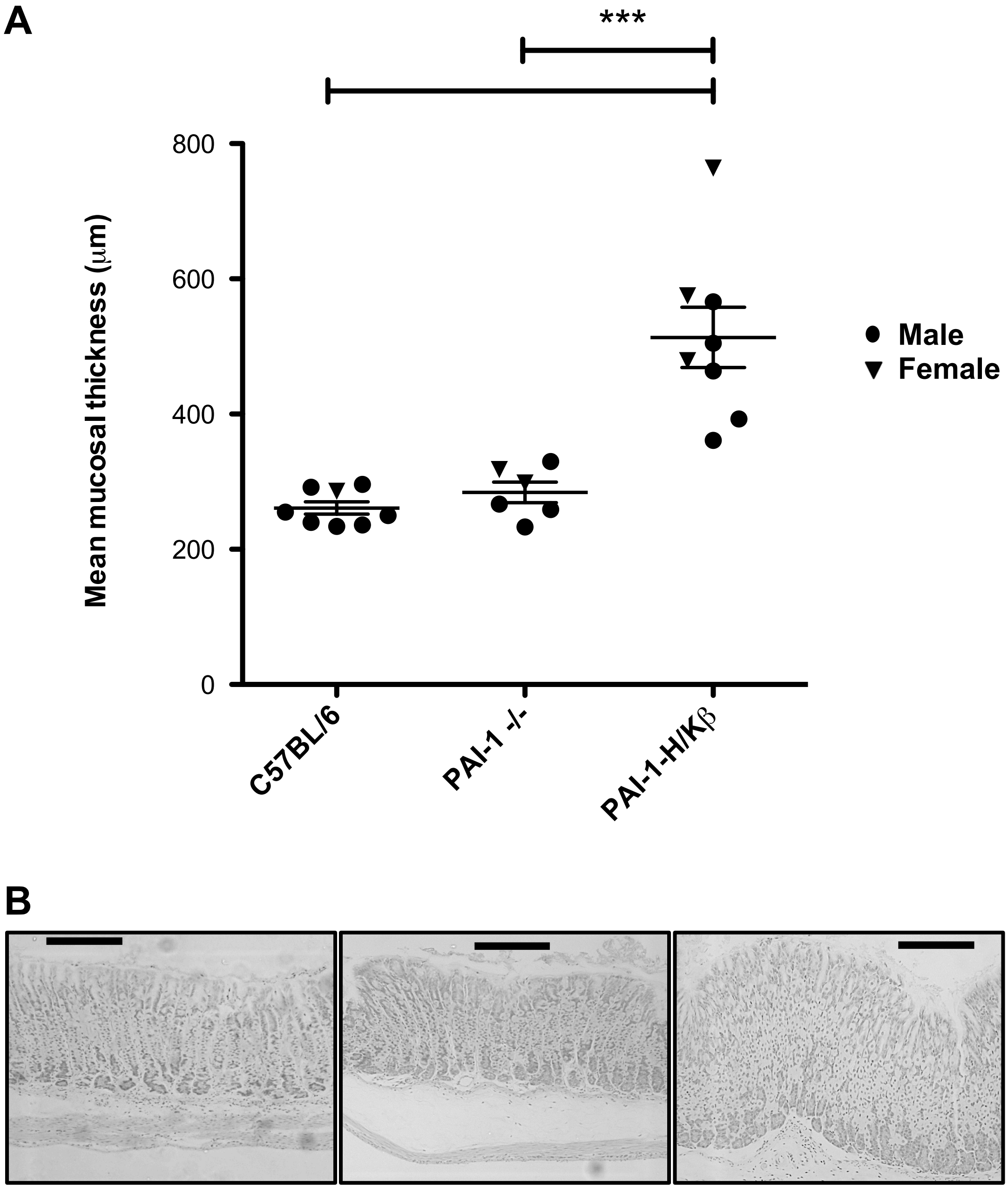
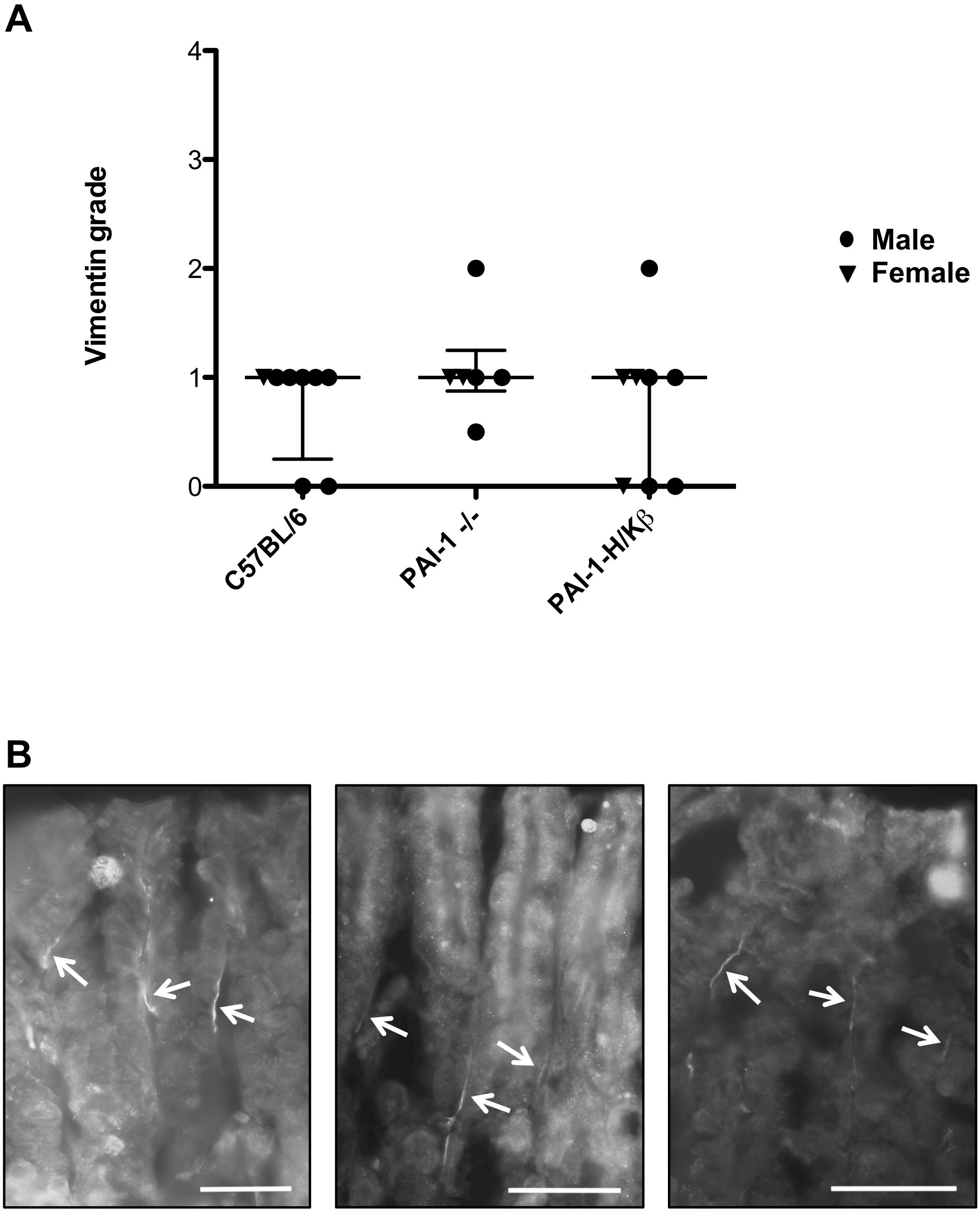


Figure 3.1. PAI-1-H/Kβ mice have significantly elevated concentrations of plasma PAI-1. The concentration of plasma PAI-1 from C57BL/6, (*n* = 5), PAI-1$^{-/-}$ (*n* = 5) and PAI-1-H/Kβ (*n* = 4) mice, aged 9-12 weeks and fed *ad libitum*, was determined by ELISA. PAI-1-H/Kβ mice had significantly higher concentrations of circulating PAI-1 compared to C57BL/6 mice. As expected, PAI-1$^{-/-}$ mice had no circulating PAI-1. Data are expressed as mean ± SEM. **P < 0.01, ***P < 0.001 (one-way ANOVA).



C57BL/6 PAI-1$^{-/-}$ PAI-1-H/Kβ

Figure 3.2. PAI-1-H/Kβ mice display mucosal thickening. A: The mean gastric corpus mucosal thickness of 53-56 week old PAI-1-H/Kβ mice was increased ~2-fold compared to both wild-type and PAI-1$^{-/-}$ mice in the same age range, with no significant difference in mucosal thickness between these latter strains. Data for individual animals are shown in addition to mean ± SEM. *** $P < 0.001$ (one-way ANOVA). **B:** Representative H&E images showing increased corpus mucosal thickness of PAI-1-H/Kβ mice (scale bars = 200μm).



C57BL/6 PAI-1$^{-/-}$ PAI-1-H/Kβ

Figure 3.3. Similar corpus mucosal myofibroblast abundance in C57BL/6, PAI-1$^{-/-}$ and PAI-1-H/Kβ mice. A: The median vimentin grade of corpus mucosa from 53-56 week old wild-type, PAI-1$^{-/-}$ and PAI-1-H/Kβ mice was comparable. Data for individual animals are shown in addition to median ± IQR. **B:** Representative images of vimentin staining in gastric corpus tissues, showing 1 – 2 myofibroblasts (arrows) within the lamina propria between each gland (scale bars = 50μm).

Wild-type, PAI-1$^{-/-}$ and PAI-1-H/Kβ corpus contained comparable numbers of parietal cells (Figure 3.4A). Parietal cells were distributed similarly in C57BL/6 and PAI-1$^{-/-}$ tissues (Figure 3.4B). PAI-1-H/Kβ corpus tissues consisted of regions showing similar patterns of parietal cell distribution as wild-type and PAI-1$^{-/-}$ tissues and regions where the parietal cell population expanded upwards along the increased gland lengths (Figure 3.4B).

3.3.4 Corpus mucosal thickness of 9-12 week old mice is dependent upon breeding environment.

To determine whether there were also morphological differences in the gastric mucosa of PAI-1-H/Kβ mice at time points earlier than 56-58 weeks, the stomachs of 9-12 week old mice were examined. The mean corpus mucosal thickness of 9-12 week old C57BL/6, PAI-1$^{-/-}$ and PAI-1-H/Kβ mice was compared and was found to be similar (Figure 3.5A). The mean corpus mucosal thickness of 9-12 week old C57BL/6 mice bred at different locations was compared. Mice from Charles River Laboratories had a significantly reduced mean mucosal thickness compared to mice bred at Harlan Laboratories and mice bred in-house (Figure 3.5B).

3.3.5 Exogenous PAI-1 attenuates growth factor stimulated gastric epithelial cell proliferation

The increased corpus mucosal thickness of PAI-1-H/Kβ mice described above raises the question of whether PAI-1 regulates epithelial cell proliferation. To test this directly, studies were undertaken using cultured gastric glands. Proliferation of isolated gastric glands was examined using an EdU-based assay.

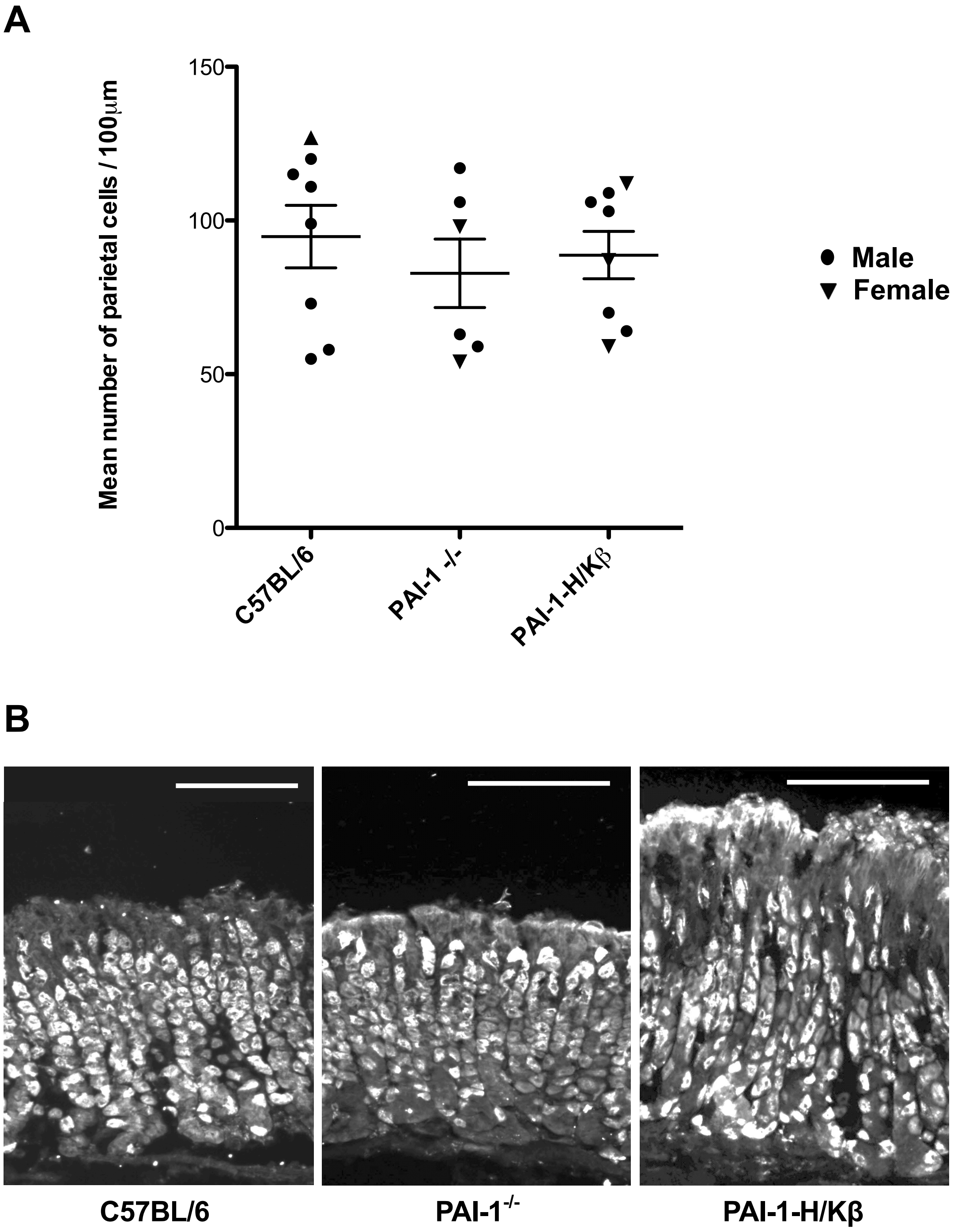


Figure 3.4. Similar parietal cell abundance in C57BL/6, PAI-1$^{-/-}$ and PAI-1-H/Kβ mice. A: The mean number of parietal cells per 100μm of full thickness corpus mucosa from 53-56 week old wild-type, PAI-1$^{-/-}$ and PAI-1-H/Kβ was similar. Data for individual animals are shown in addition to mean ± SEM. **B:** Representative images of H/K ATPase immunopositive cells within gastric corpus mucosal tissue from wild-type, PAI-1$^{-/-}$ and PAI-1-H/Kβ mice (scale bars = 200μm).

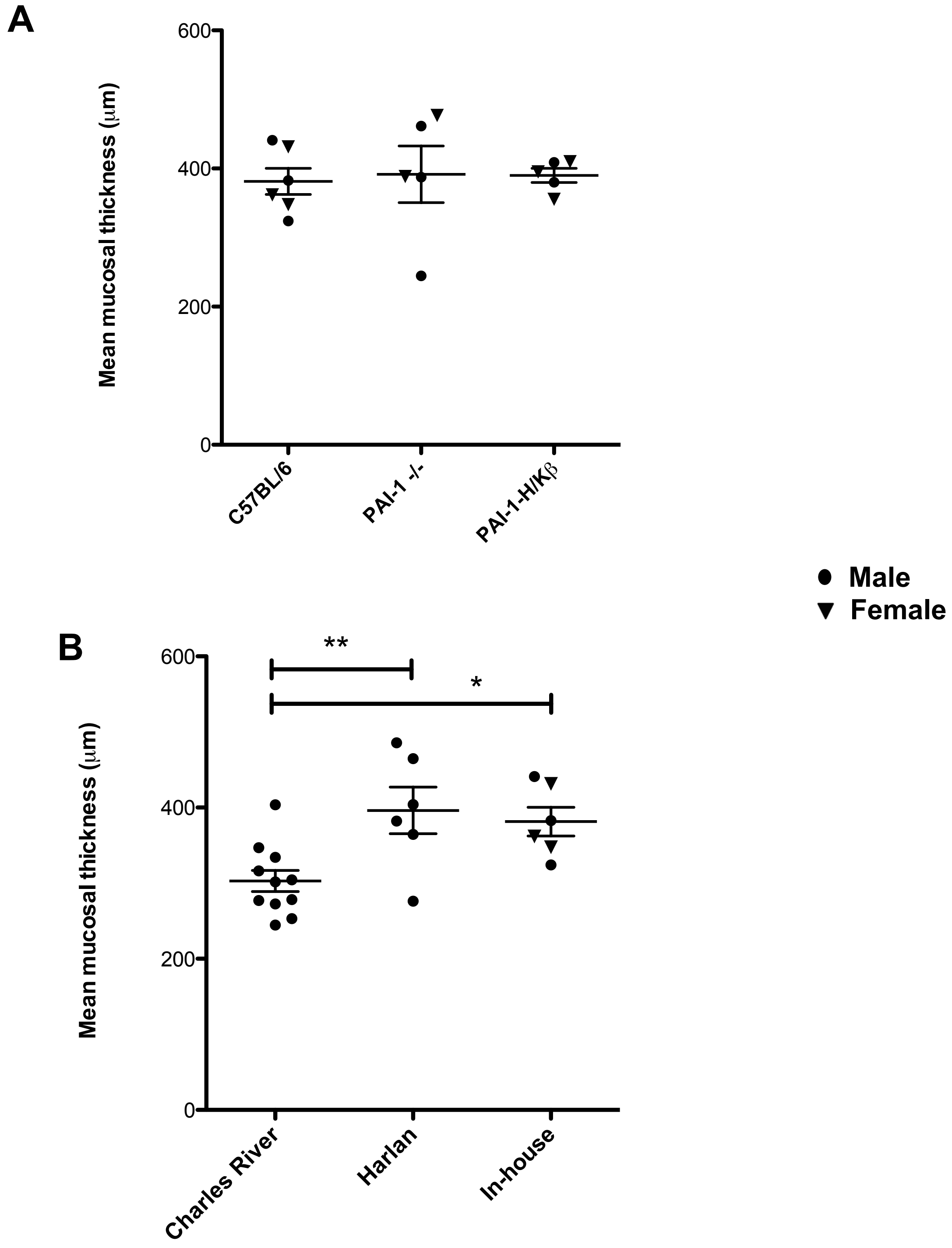


Figure 3.5. Corpus mucosal thickness of mice aged 9-12 weeks is influenced by breeding location. A: Similar corpus mucosal thickness of 9-12 week old C57BL/6, PAI-$1^{-/-}$ and PAI-1-H/Kβ mice bred in-house. **B:** C57BL/6 mice purchased from Charles River Laboratories had significantly reduced corpus mucosal thickness compared to mice purchased from Harlan Laboratories and mice bred in-house. Data for individual animals are shown in addition to mean ± SEM. * $P < 0.05$, ** $P < 0.01$ (one-way ANOVA).

Contaminating myofibroblasts were initially identified in gastric gland cultures by positive vimentin staining and were found to be consistently rare, at no more than 5% of the total cell population. Basal EdU incorporation into cycling C57BL/6, PAI-$1^{-/-}$ and PAI-1-H/Kβ epithelial cells within the isolated gastric glands was comparable (Figure 3.6B).

Gastric glands isolated from C57BL/6, PAI-$1^{-/-}$ and PAI-1-H/Kβ mice were treated with 20nM PAI-1, with or without 1ng/ml EGF or 100ng/ml IGF-II, for a total period of 20 hours, including 2 hours incubation with 10μM EdU. Exogenous PAI-1 had no effect upon EdU incorporation in any mouse strain (Figure 3.7A-F). Exogenous EGF stimulated EdU incorporation into wild-type and PAI-$1^{-/-}$ gastric glands (Figure 3.7A & C). Exogenous IGF-II also stimulated EdU incorporation into gastric glands from all strains (Figure 3.7B, D & F). Exogenous PAI-1 attenuated EGF- and IGF-II-stimulated EdU incorporation into wild-type gastric glands (Figure 3.7A & B). PAI-1 attenuated EGF-stimulated EdU incorporation into PAI-$1^{-/-}$ gastric glands (Figure 3.7C) and IGF-II-stimulated EdU incorporation into PAI-1-H/Kβ gastric glands (Figure 3.7F).

3.4 Discussion

The work in this chapter describes the gastric corpus mucosal phenotype of PAI-$1^{-/-}$ and PAI-1-H/Kβ mice compared to wild-type, in order to determine the role of PAI-1 in shaping gastric mucosal morphology. The data revealed that PAI-1-H/Kβ mice develop age-dependent increases in corpus mucosal thickness, independent of changes in parietal cell numbers. This implies that another corpus mucosal cell population is

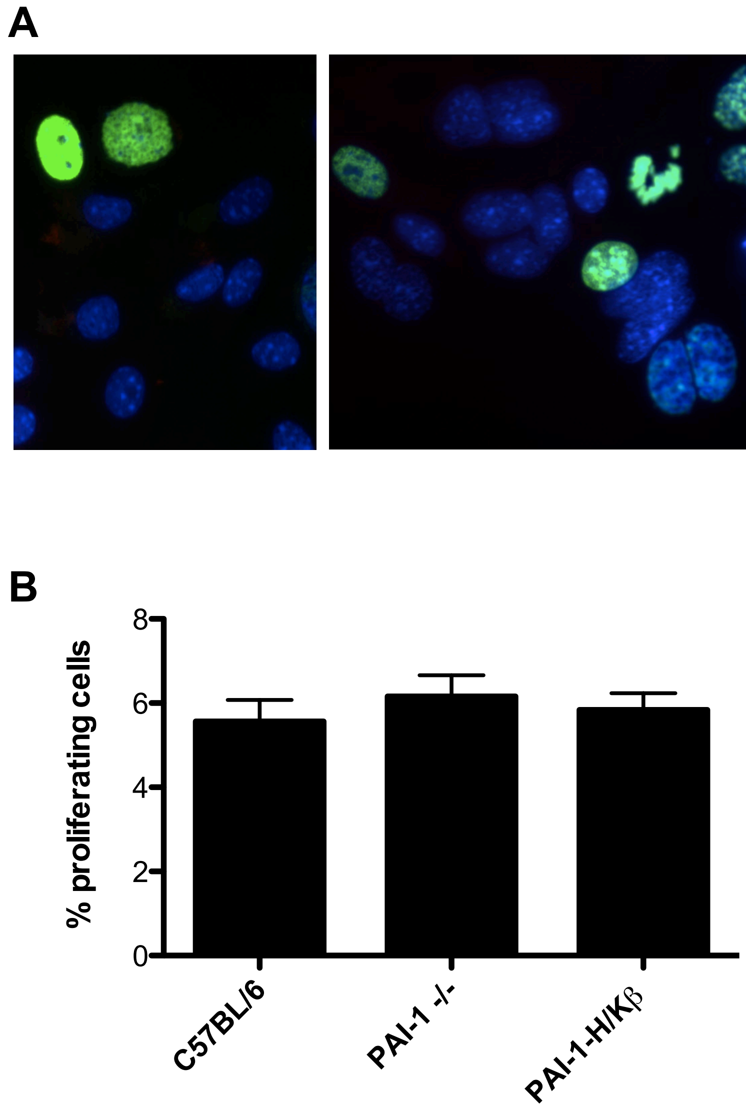


Figure 3.6. Isolated gastric glands from C57BL/6, PAI-1$^{-/-}$ and PAI-1-H/Kβ mice exhibit similar incorporation of EdU. A: Examples of EdU positive (green nuclei) and EdU negative (blue nuclei) gastric epithelial cells. EdU incorporation was calculated as the percentage of nuclei that were EdU positive. **B:** Basal incorporation of EdU into isolated gastric glands from C57BL/6 (n = 12), PAI-1$^{-/-}$ (n = 14) and PAI-1-H/Kβ (n = 15) mice was similar. Data are expressed as mean ± SEM.

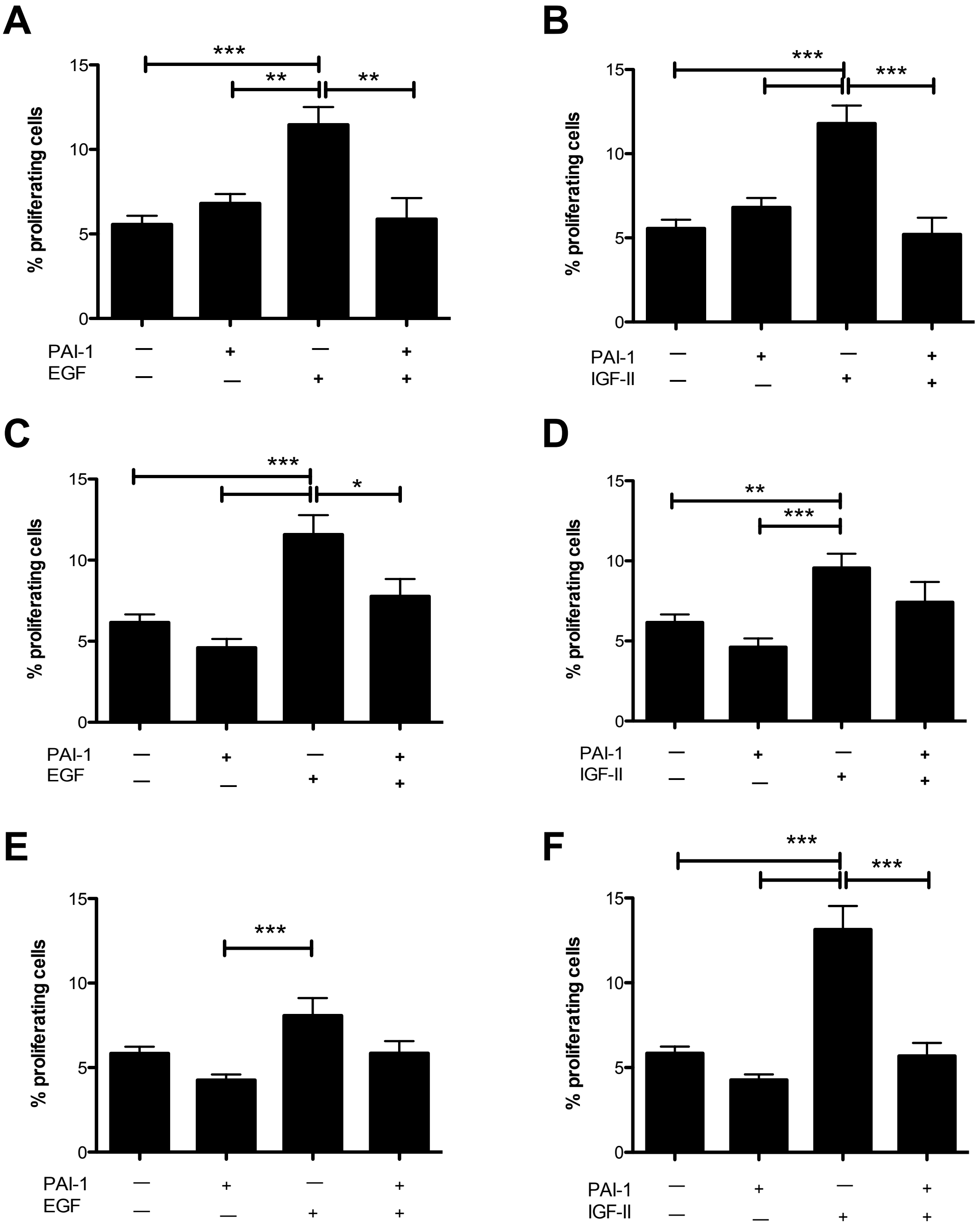


Figure 3.7. Basal EdU incorporation into C57BL/6, PAI-1$^{-/-}$ and PAI-1-H/Kβ gastric glands is unaltered by exogenous PAI-1. A & B: Effect of PAI-1 (*n* = 7), EGF (*n* = 7), EGF plus PAI-1 (*n* = 3), IGF-II (*n* = 9) and IGF-II plus PAI-1 (*n* = 4) on EdU incorporation into wild-type gastric glands, compared to untreated glands (*n* = 12). EGF and IGF-II significantly stimulated EdU incorporation. Exogenous PAI-1 did not alter basal EdU incorporation but attenuated growth factor-stimulated EdU incorporation. **C & D** Comparison repeated using PAI-1$^{-/-}$ glands (*n* = 4-14). Exogenous PAI-1 attenuated EGF-stimulated EdU incorporation. **E & F**: Comparison repeated using PAI-1-H/Kβ glands (*n* = 6-15). Exogenous PAI-1 attenuated IGF-II-stimulated EdU incorporation. Concentrations used: 20nM PAI-1, 1ng/ml EGF and 100ng/ml IGF-II, each for 20 hours total incubation period. Data are expressed as mean ± SEM. $*P < 0.05$, $**P < 0.01$, $***P < 0.001$ (one-way ANOVA).

expanded in PAI-1-H/Kβ tissue. Further immunohistochemical characterisation of the major epithelial cell populations, including ECL cells, chief cells, mucous neck cells, proliferating isthmal cells and pit cells, in corpus tissue of PAI-1-H/Kβ mice compared to C57BL/6 and PAI-1$^{-/-}$ mice would provide a more complete understanding of the histological basis of increased mucosal thickness in response to elevated gastric expression of PAI-1. The concentration of plasma PAI-1 in each mouse strain was also determined. Predictably, PAI-1$^{-/-}$ mice had no detectable circulating PAI-1. PAI-1-H/Kβ mice had circulating PAI-1 concentrations approximately 3-fold higher than wild-type. The mean concentration of plasma from C57BL/6 and PAI-1-H/Kβ mice was of a similar order as reported in previous studies (Kenny *et al.*, 2013a).

In terms of epithelial cell dynamics underlying increases in mucosal thickness observed in PAI-1-H/Kβ corpus tissue, one possibility is that apoptosis is suppressed in response to increased gastric PAI-1 expression. PAI-1 has been shown to negatively-regulate apoptosis of various normal and malignant cell populations, including breast epithelial cells, human umbilical vein endothelial cells (HUVECs), vascular smooth muscle cells (VSMCs), neuronal cells, neutrophils, lung fibroblasts, prostate cancer cells, promyelocytic leukaemia cells and fibrosarcoma cells (Kwaan *et al.*, 2000; Soeda *et al.*, 2001; Chen *et al.*, 2004; Lademann *et al.*, 2005; Soeda *et al.*, 2006; Suzuki *et al.*, 2008; Romer *et al.*, 2008; Horowitz *et al.*, 2008; Zmijewski *et al.*, 2011; Zhang *et al.*, 2013). Similarly, increased expression of PAI-2 by AGS gastric cancer cells is associated with a reduction in *H. pylori*-induced apoptosis (Varro *et al.*, 2004). However, PAI-1 has also demonstrated a pro-apoptotic effect on various cell populations (Al-Fakhri *et al.*, 2003; Xiang *et al.*, 2005; Balsara *et al.*, 2006; Chen *et al.*, 2008; Hu *et al.*, 2009; Wu *et al.*, 2009). Furthermore, the basal rate of non-surface corpus epithelial cell apoptosis is already very low (Court *et al.*, 2003; Przemeck *et al.*, 2007; Przemeck *et al.*, 2008; Duckworth *et al.*, 2012). However, increased mucosal thickness develops over 53-56 weeks, so further small reductions in apoptosis could accumulate to increase mucosal thickness over this time period. This warrants further investigation.

An alternative hypothesis for increased corpus mucosal thickness in PAI-1-H/Kβ mice is that epithelial cell proliferation is increased in PAI-1-H/Kβ corpus tissue. This hypothesis is supported by various studies examining the relationship between PAI-1 expression and proliferation rates *in vivo*. In a murine arterial injury model, PAI-1 expression correlated with VSMC proliferation and neointimal formation, proposed to be mediated by the proliferative effects of fibrinogen accumulation (Suzuki *et al.*, 2008). Similarly, PAI-1 expression correlated with *in vivo* proliferation of murine mammary carcinoma cells and fibrosarcoma cells, and consequently with tumourigenesis (Gutierrez *et al.*, 2000; Jing *et al.*, 2012). Conversely, increased expression of PAI-1 was shown to repress prostate cancer tumourigenesis in athymic mice (Soff et al, 1995). Although PAI-1 negatively regulated hepatocyte and cholangiocyte proliferation in anti-Fas and bile duct ligation models of liver injury, PAI-1 promoted proliferation in the more severe carbon tetrachloride liver injury model (Shimizu *et al.*, 2001; Wang *et al.*, 2005; von Montford *et al.*, 2010). These contrary results indicate both tissue-specific and microenvironment-specific roles for PAI-1 in proliferation. Future studies to determine proliferation in the gastric corpus mucosa of PAI-1-H/Kβ mice compared to PAI-1$^{-/-}$ and C57BL/6 mice, utilising assays examining *in vivo* incorporation of EdU into gastric epithelial cells, would provide clarification on the role of proliferation in determining the gastric mucosal phenotype of PAI-1-H/Kβ mice.

The finding that corpus mucosal thickness of wild-type mice aged 9-12 weeks, which had been bred in-house, was increased compared to mice aged 53-56 weeks, which had been bred externally, raised the question of whether differences in mucosal thickness observed in these studies might have been determined by factors relating to the breeding environment. A direct comparison revealed that mice bred at one source had a significantly reduced mean mucosal thickness compared to mice bred at 2 other sources, suggesting that the breeding environment influenced the gastric phenotype. This is supported by variations in the thickness of corpus mucosal tissue of C57BL/6 mice reported in previous studies, ranging from 278μm to 682μm (Farrell *et al.*, 2002; Aihara *et al.*, 2003; Fukui *et al.*, 2006; Park *et al.*, 2008; Van der Burg *et al.*, 2011; Lu *et al.*, 2011).

Variations in the gastric phenotype of mice bred at separate locations could be due to differences in gut microbiota. A previous study showed that gnotobiotic mice had shortened gastric glands compared to specific pathogen free mice and mice colonised by altered Schaedler flora (Schmitz *et al.*, 2011). This was associated with a reduction in the number of proliferating cells and loss of the prezymogenic cell population. Future studies could examine gastric morphological features of gnotobiotic C57BL/6, PAI-1$^{-/-}$ and PAI-1-H/Kβ mice, eliminating the confounding factor of commensal bacteria in the comparative analysis of gastric phenotypes. However, comparisons between 53-56 week old PAI-1$^{-/-}$ and PAI-1-H/Kβ mice in this study remain valid, given that these mice were bred at the same location over many generations.

In order to elucidate the mechanism by which either local increases in corpus mucosal PAI-1 expression or elevated circulating PAI-1 stimulates mucosal thickening, the possibility that PAI-1 directly stimulates epithelial cell proliferation was investigated using cultured isolated gastric glands. Proliferation of both PAI-1$^{-/-}$ and PAI-1-H/Kβ epithelial cells was similar to wild type, as determined by EdU incorporation, implying that neither autocrine nor paracrine PAI-1 secretion on its own stimulates epithelial cell proliferation. Furthermore, exogenous PAI-1 had no effect on gastric epithelial cell proliferation. Although PAI-1 did not affect basal epithelial cell proliferation, exogenous PAI-1 did attenuate growth factor stimulated proliferation.

A direct anti-proliferative role of PAI-1 has been reported in a number of previous *in vitro* studies. PAI-1 was shown to inhibit basal and growth factor-stimulated proliferation of isolated mouse aortic endothelial cells, via interactions of the PAI-1-uPA-uPAR complex with LRP, and modulation of the PI3K/Akt signalling pathway (Ploplis *et al.*, 2004; Balsara *et al.*, 2006). Similarly, PAI-1 supressed proliferation of fibroblasts via the PI3K/Akt signalling pathway (Kortlever *et al.*, 2006; Ardite *et al.*, 2012). Exogenous PAI-1 inhibited proliferation of lung cancer cells and HUVECs, but not prostate cancer cells (Chorostowska-Wynimko *et al.*, 2010). Contrary to the reports of anti-proliferative effects of PAI-1 on cell populations, the PAI-1-uPA complex stimulated proliferation of a gastric adenocarcinoma line via increased very low density lipoprotein receptor expression, and isolated VSMCs over-expressing PAI-1 exhibited increased proliferation, via activation of NF-κB and MAPK signalling pathways (Chen *et al.*, 2006; Di *et al.*, 2010).

The varied effects of PAI-1 on proliferation of cell populations highlights the importance of cell phenotype, the microenvironment and activated signalling cascades in determining the role of PAI-1 in regulating proliferation. Furthermore, the effects of PAI-1 on modulating cell and tissue dynamics are highly dose-dependent. This may explain the observation that exogenous PAI-1 suppressed IGF-II-stimulated proliferation of wild-type gastric glands, whilst increased expression of PAI-1 in the gastric mucosa of PAI-1-H/Kβ mice was not sufficient to elicit such an effect. EGF signalling pathways may be more sensitive to the inhibitory effects of PAI-1, since EGF failed to stimulate significant proliferation of PAI-1-H/Kβ gastric glands.

Since PAI-1 did not directly stimulate proliferation of gastric epithelial cells, this raises the question as to whether PAI-1 mediates an indirect proliferative mechanism in the gastric mucosa. One possibility is that hyperphagia in PAI-1-H/Kβ mice stimulates proliferation of the gastric mucosa over 53-56 weeks. Feeding is known to stimulate proliferation of the corpus mucosa, via the trophic effects of gastrin (Johnson, 1977; Ohning *et al.*, 1996). However, plasma gastrin of PAI-1-H/Kβ mice is not significantly different to that of wild-type mice, so increases in mucosal thickness are unlikely to be gastrin-dependent (Kenny *et al.*, 2013a).

An alternative hypothesis is that PAI-1 regulates myofibroblast activity, modulating myofibroblast-epithelial interactions to stimulate proliferation. Previous studies have identified a reciprocal epithelial-myofibroblast interaction, mediated by epithelial cell derived MMP-7, that stimulates proliferation of both gastric epithelial cells and myofibroblasts in hypergastrinaemia and in *H. pylori* infection (McCaig *et al.*, 2006; Varro *et al.*, 2007). IGF-II was identified as a myofibroblast-derived growth factor stimulating proliferation of both epithelial cells and myofibroblasts. Similar to MMP-7, gastric epithelial cell expression of PAI-1 is increased by gastrin, and gastric PAI-1 is elevated in hypergastrinaemia and in *H. pylori* infection (Herszenyi *et* al, 1997; Kenny *et al.*, 2008; Keates *et al.*, 2008; Ikeda *et al.*, 2009; Norsett *et al.*, 2011). The abundance of corpus mucosal myofibroblasts was investigated to establish whether PAI-1 mediates a similar proliferative response. Increases in corpus mucosal thickness were independent of changes in myofibroblast numbers in PAI-1-H/Kβ mice. This does not exclude the possibility of altered gene expression by PAI-1-H/Kβ myofibroblasts; the effect of PAI-1 on myofibroblast gene expression is examined in chapter 4 of this thesis.

In summary, the data presented in this chapter suggest that PAI-1 might act via indirect mechanisms to stimulate proliferation in the gastric corpus mucosa, independent of changes in parietal cell and myofibroblast abundance. Potential mechanisms by which PAI-1 might regulate myofibroblast-epithelial interactions to modulate epithelial cell proliferation will be investigated in the next chapter. Other aspects of the gastric phenotype of PAI-1$^{-/-}$ and PAI-1-H/Kβ mice require investigation, including responses to chronic *Helicobacter* infection and gastric mucosal protection; these will be investigated in chapters 5 and 6 respectively.

3.5 Conclusions

1. Plasma PAI-1 concentrations are elevated in PAI-H/Kβ mice.
2. PAI-1-H/Kβ mice develop age-dependent thickening of the gastric mucosa, independent of changes in parietal cell numbers or corpus mucosal myofibroblast abundance.
3. The breeding environment influences the corpus mucosal thickness of C57BL/6 mice.

4. Endogenous PAI-1 expression has no effect upon gastric epithelial cell proliferation.
5. Exogenous PAI-1 has no effect upon basal gastric epithelial cell proliferation, but attenuates growth factor-stimulated proliferation.

CHAPTER 4

REGULATION OF GASTRIC MUCOSAL MYOFIBROBLAST-EPITHELIAL INTERACTIONS BY PAI-1

4.1 Introduction

Myofibroblasts reside within the lamina propria of the gastrointestinal mucosa, interacting with epithelial cells and other stromal cells, via direct cell-cell communication and paracrine secretions, and also with the ECM (Valentich & Powell, 1994; Powell *et al.*, 1999; Wu *et al.*, 1999; Powell *et al.*, 2011). Gastrointestinal myofibroblasts are thus important regulators of epithelial cell dynamics, integrating signals between epithelial cells and other subepithelial mesenchymal cell populations (Valentich & Powell, 1994; Powell *et al.*, 2005). These interactions define the mucosal microenvironment. Proteins secreted by myofibroblasts influence the activity of epithelial cells, in physiological and pathophysiological settings. The phenotype of myofibroblasts is reciprocally determined by the microenvironment in which they reside.

A particularly well-characterised mediator of epithelial-myofibroblast interactions in the gastric mucosa is MMP-7. Expression of MMP-7, which is secreted by epithelial cells, is increased in response to *H. pylori* infection and hypergastrinaemia (Wroblewski *et al.*, 2003; Varro *et al.*, 2007). Subsequently myofibroblast and epithelial cell proliferation, and myofibroblast migration, is stimulated via MMP-7 release of IGF-II from IGF binding protein (IGFBP)-5, both of which are released by myofibroblasts (Hemers *et al.*, 2005; McCaig *et al.*, 2006; Varro *et al.*, 2007). Another important mediator of gastric epithelial-mesenchymal interactions is TGF-β, which increases myofibroblast abundance via transdifferentiation of fibroblasts, in the primary gastric carcinoma microenvironment, and mesothelial cells in peritoneal metastases (Fuyuhiro *et al.*, 2011; Lv *et al.*, 2013). Consequently, migration, invasion and metastasis of cancer cells are increased (Fuyuhiro *et al.*, 2010; Fuyuhiro *et al.*, 2012; Lv *et al.*, 2013).

The role of the microenvironment in determining the phenotype of gastric myofibroblasts is highlighted by previous studies showing distinct differences in gene expression, secretomes and dynamics of CAMs compared to ATMs and NTMs (Holmberg *et al.*, 2012; Balabanova, 2012; Holmberg *et al.*, 2013). These changes are functionally significant, in terms of influencing epithelial cell dynamics. The secretomes of CAMs stimulate increased proliferation, migration and invasion of gastric cancer cells, in part due to a decrease in TGFβ-induced gene-h3, leading to increased IGF-II activity, and increased MMP activity (Holmberg *et al.*, 2012; Holmberg *et al.*, 2013).

The results of the previous chapter indicated that PAI-1 might positively regulate proliferation of gastric epithelial cells via an indirect mechanism. One possibility is that PAI-1 influences myofibroblast gene expression, causing secretion of proteins that modulate gastric epithelial cell dynamics. This chapter compares the phenotypes of myofibroblasts in gastric mucosal microenvironments free of PAI-1 compared to those in PAI-1-positive microenvironments.

4.1.1 Aims and objectives

The aim of the work in this chapter was to characterise the transcriptomes of gastric myofibroblasts from wild-type and PAI-1$^{-/-}$ mice, with a view to identifying myofibroblast-secreted proteins that might be responsible for differences in gastric mucosal function in the two strains. The specific objectives were to:

1. compare global gene expression profiles of cultured gastric myofibroblasts from C57BL/6 and PAI-1$^{-/-}$ mice;
2. characterise the phenotypes of C57BL/6 and PAI-1$^{-/-}$ antral mucosal myofibroblasts *in situ*;
3. characterise the phenotypes of dissociated C57BL/6 and PAI-1$^{-/-}$ antral myofibroblasts;
4. examine the functional significance of differences in the phenotypes of C57BL/6 and PAI-1$^{-/-}$ antral myofibroblasts.

4.2 Methods

4.2.1 Gene expression microarray analysis

Normalisation and analysis of raw signal intensities generated by the GeneChip® Mouse Genome 430 2.0 arrays was accomplished using Affymetrix MAS5.0 summary algorithm in GeneSpring GX 10. Three microarrays were performed for each mouse strain, using material from single animals for each sample. Data were grouped and averaged by mouse strain, to compare global gene expression by cultured antral C57BL/6 and PAI-1$^{-/-}$ myofibroblasts, described in section 2.13. These myofibroblasts had been passaged no more than 4 times. Only probe sets that were present in all samples of C57BL/6 or PAI-1$^{-/-}$ myofibroblasts were included in the comparative analysis. Differentially expressed probe sets were identified using an unpaired *t*-test with Benjamini-Hochberg correction (significant at $P < 0.05$), using cut-off value of ±3-fold change, and the corresponding genes and gene products were identified by putting the entity list through MetaRodent™. The Gene Ontology database (www.geneontology.org) was used to identify differentially expressed transcripts with an extracellular gene product.

4.2.2 Immunofluorescence

Immunohistochemistry was performed on mouse antral tissue sections, as described in section 2.9. Immunocytochemistry was performed on dissociated mouse or cultured human antral myofibroblasts, according to the methods described in section 2.10. Mouse antral myofibroblasts were isolated and acutely cultured, as described in section 2.6, by dividing denuded antral pieces from each animal into 2 wells of a 24 well plate. Mouse antral myofibroblasts for immunocytochemistry were not passaged. Human myofibroblasts were passaged no more than 10 times.

4.2.3 Dipeptidyl peptidase 4 activity assays

Human NTMs were passaged no more than 12 times and were plated at the required number in a 94 well plate. DPP4 activity of human myofibroblasts or mouse plasma was assayed as described in section 2.14.

4.3 Results

4.3.1 Gastric myofibroblasts from PAI-1 null mice have a global gene expression profile distinct from wild-type gastric myofibroblasts

Whole mouse genome microarrays were used to compare global gene expression profiles of wild-type and PAI-1$^{-/-}$ gastric myofibroblasts, in order to identify differentially-regulated transcripts. Quality control analysis confirmed that the arrays could be reliably compared to each other (Figure 4.1). Principal component analysis showed distinct clustering of samples based on mouse strain (Figure 4.2A). The array data was filtered to include only probe-sets present in all samples of C57BL/6 or PAI-1$^{-/-}$ myofibroblasts for further analyses, producing a data-set of 22311 entities. The normalised signal intensity of each entity was grouped and averaged by mouse strain, revealing a distinct profile of gene expression by wild-type and PAI-1$^{-/-}$ gastric myofibroblasts (Figure 4.2B).

A list of differentially expressed probe-sets was generated comparing mean normalised signal intensities of C57BL/6 and PAI-1$^{-/-}$ samples. Entities were considered differentially expressed if $P < 0.05$ (unpaired *t*-test; Benjamini-Hochberg correction) and fold-change was ≥ 3 or ≤ -3, to increase the power of the data analysis and generate a workable list of transcripts. A fold change value of ±3 was considered sufficiently stringent against the detection of false-positives in the lower range of absolute expression values, whilst being sensitive enough to detect significant changes in highly expressed transcripts. After putting the entity list through MetaRodent™, a total of 1767 gene products were identified as being

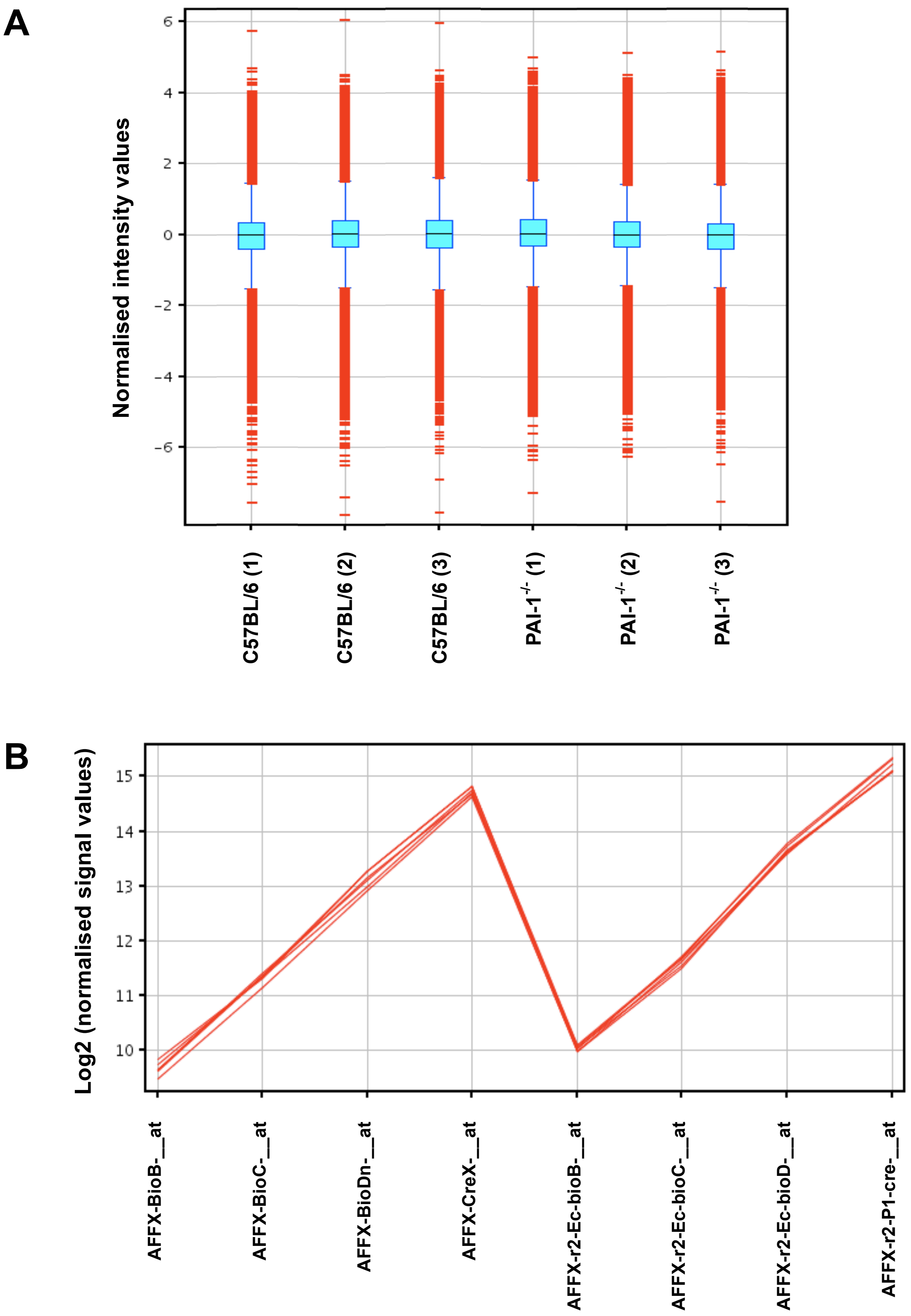


Figure 4.1. Quality control analysis indicates similar performance across all microarrays. A: Box whisker plot of normalised signal intensities across the 6 microarrays showed a similar distribution of signal intensities for each sample. **B:** Normalised signal intensities of hybridisation control entities were similar across all microarrays. A single red line represents each of the 6 samples and 8 control entities are plotted.

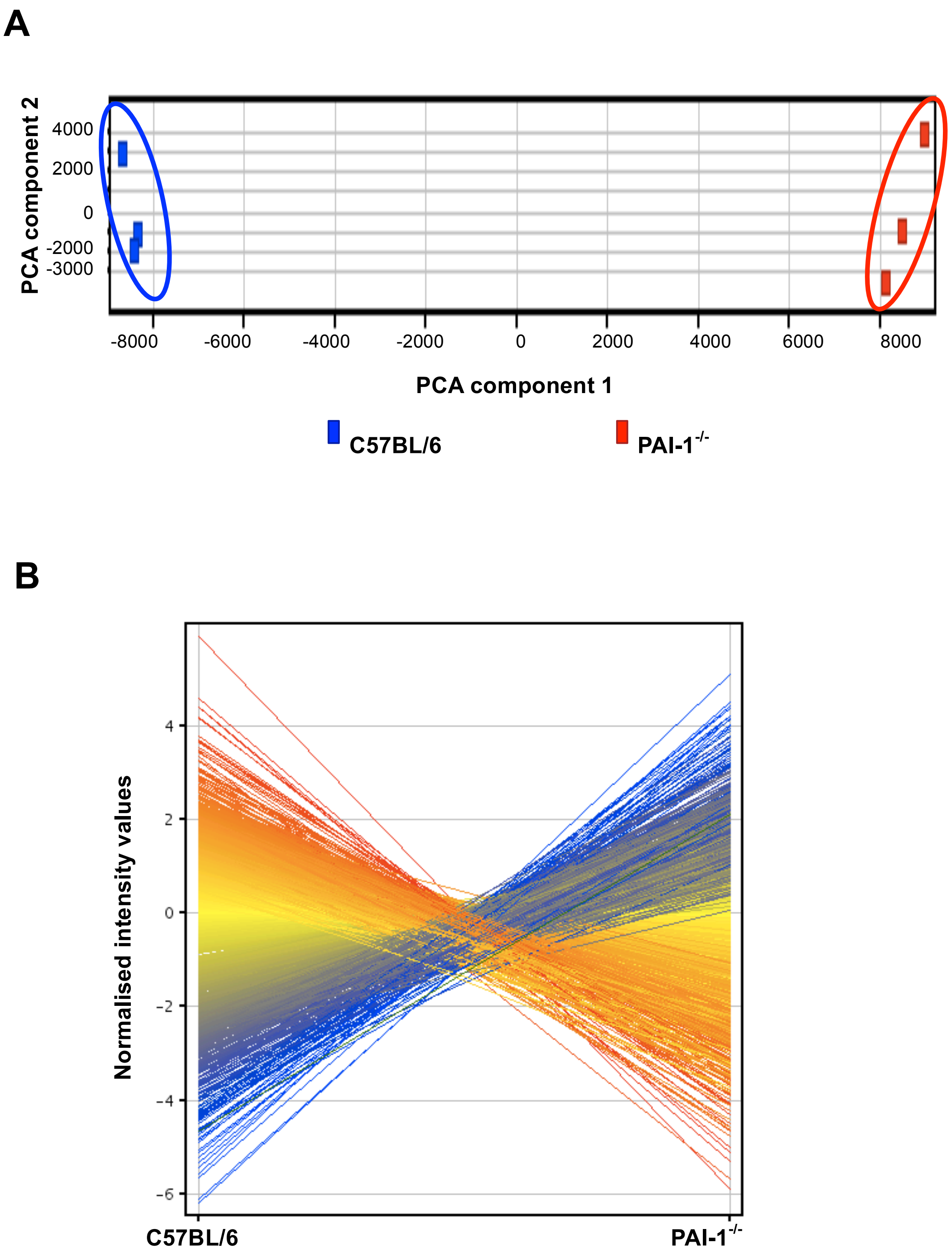


Figure 4.2. Gastric myofibroblasts from C57BL/6 and PAI-1$^{-/-}$ mice show distinct global gene expression profiles. A: Principal component analysis showed clustering of samples by mouse strain, based on separation by principal component 1. **B:** Profile plot of normalised signal intensities of individual entities, grouped by mouse strain and averaged, showed distinct gene expression profiles by gastric myofibroblasts from C57BL/6 and PAI-1$^{-/-}$ mice. The data were filtered from whole microarray data to include only entities present in all samples of C57BL/6 or PAI-1$^{-/-}$ myofibroblasts.

differentially expressed. The relative expression of transcripts encoding known markers of stromal cell differentiation was analysed, revealing 8 differentially expressed markers by C57BL/6 and PAI-1$^{-/-}$ gastric myofibroblasts (Table 4.1). In order to identify potential PAI-1 dependent modulators of the tissue microenvironment, the dataset was also filtered by gene ontology to generate a list of 223 differentially expressed transcripts with an extracellular gene product. The 20 most differentially expressed transcripts are shown in Table 4.2.

4.3.2 Antral myofibroblasts exist as a heterogeneous population of vimentin- and desmin-expressing cells

In order to validate the data derived from gene expression microarrays, the expression of two known stromal lineage markers in mouse antral sections and in dissociated mouse antral myofibroblasts was investigated by immunofluorescence. Although there were remaining stocks of cultured C57BL/6 myofibroblasts used in the microarrays, stocks of PAI-1$^{-/-}$ myofibroblasts were used up during extraction of RNA for microarray analysis. For this reason, freshly dissociated myofibroblasts were prepared for validation studies. Vimentin was selected as a routine myofibroblast identifier and desmin was selected on the basis of a large fold-change difference in mRNA expression by PAI-1$^{-/-}$ antral myofibroblasts relative to C57BL/6 antral myofibroblasts (Table 4.1). The expression of desmin mRNA had in fact been flagged as absent in all samples of C57BL/6 myofibroblasts using Affymetrix MAS5.0 summary algorithm in GeneSpring GX 10. Characterisation of α-SMA expression by immunofluorescence was also attempted, but this was uninformative owing to non-specific staining by the secondary antibody. There

Affymetrix ID	Gene Symbol	Gene Product	Fold-change (PAI-1$^{-/-}$: C57BL/6)
1426731_at	**Des**	Desmin	75
1422340_a_at	**Actg2**	Gamma-smooth muscle actin	20
1444105_at	**Acta2**	Alpha-smooth muscle actin	15
1437218_at	**Fn1**	Fibronectin	3.1
1433768_at	**Palld**	Palladin	2.3
1450641_at	**Vim**	Vimentin	1.8
1419309_at	**Pdpn**	Podoplanin	1.7
1420653_at	**Tgfb1**	Transforming growth factor beta-1	1.5
1417439_at	**Cd248**	Endosialin	-1.3
1446951_at	**P4ha3**	Prolyl 4-hydroxylase subunit alpha-3	-3.3
1450757_at	**Cdh11**	Cadherin-11	-3.8
1417234_at	**Mmp11**	Stromelysin 3 (Matrix metalloproteinase-11)	-4.1
1416298_at	**Mmp9**	Matrix metalloproteinase-9	-11

Table 4.1. Expression of transcripts encoding markers of stromal cell differentiation. There was differential expression of a number of myofibroblast markers by cultured PAI-1$^{-/-}$ antral myofibroblasts relative to C57BL/6 antral myofibroblasts (unpaired *t*-test, Benjamini-Hochberg correction, $P < 0.05$). Of the transcripts shown, genes that were differentially expressed by less than 3-fold difference in either direction were considered to be uninformative.

Affymetrix ID	**Gene Symbol**	**Gene Product**	**Fold-change (PAI-1$^{-/-}$: C57BL/6)**
1425546_a_at	**Trf**	Serotransferrin	626
1452004_at	**Calca**	Calcitonin gene-related peptide 1	209
1449319_at	**Rspo1**	R-spondin-1	156
1452065_at	**Vstm2a**	V-set and transmembrane domain-containing protein 2A	126
1416697_at	**Dpp4**	Dipeptidyl peptidase 4	109
1460238_at	**Msln**	Mesothelin	71
1460049_s_at	**1500015O10 Rik**	Augurin	-67
1452968_at	**Cthrc1**	Collagen triple helix repeat-containing protein 1	-68
1447862_x_at	**Thbs2**	Thrombospondin-2	-71
1439260_a_at	**Enpp3**	Ectonucleotide pyrophosphatase/phosphodiesterase family member 3	-76
1448147_at	**Tnfrsf19**	Tumour necrosis factor receptor superfamily member 19	-82
1430313_at	**Adamtsl1**	ADAMTS-like protein 1	-93
1417936_at	**Ccl9**	C-C motif chemokine 9	-102
1448136_at	**Enpp2**	Ectonucleotide pyrophosphatase/phosphodiesterase family member 2	-120
1418511_at	**Dpt**	Dermatopontin	-126
1419083_at	**Tnfsf11**	Tumour necrosis factor ligand superfamily member 11	-136
1419728_at	**Cxcl5**	C-X-C motif chemokine 5	-145
1455893_at	**Rspo2**	R-spondin-2	-164
1450708_at	**Scg2**	Secretogranin-2	-688
1416211_a_at	**Ptn**	Pleiotrophin	-3667

Table 4.2. Top 20 transcripts encoding extracellular proteins differentially expressed by cultured PAI-1$^{-/-}$ antral myofibroblasts compared to cultured wild type antral myofibroblasts. There were a total of 223 genes, classified as ‘extracellular’ by gene product ontology, that were significantly differentially expressed by PAI-1$^{-/-}$ antral myofibroblasts compared to wild type (unpaired *t*-test, Benjamini-Hochberg correction, $P < 0.05$, fold-change ≥ 3 or ≤ -3). The table shows the top 20 transcripts, ranked by fold change in either direction, with ‘extracellular’ annotation up- or down-regulated in PAI-1$^{-/-}$ myofibroblasts relative to wild type.

were two distinct populations of vimentin- and desmin-expressing cells with typical myofibroblast-like spindle-shaped morphology within the lamina propria of both C57BL/6 and PAI-1$^{-/-}$ antral sections (Figure 4.3). Cells expressing both vimentin and desmin were rare and expressed one of the signals only weakly. Dissociated antral myofibroblasts from PAI-1$^{-/-}$ mice similarly expressed vimentin and desmin to varying degrees (Figure 4.4).

4.3.3 Secretogranin-2 and dipeptidyl peptidase 4 are expressed by dissociated PAI-1$^{-/-}$ antral myofibroblasts

To further validate the microarray data, expression of proteins encoded by differentially expressed transcripts was determined in mouse antral sections and dissociated mouse antral myofibroblasts from PAI-1$^{-/-}$ mice. Despite strenuous efforts, attempts to dissociate myofibroblasts from C57BL/6 mice were unsuccessful, so it was not possible to perform validations on wild-type myofibroblasts. Secretogranin-2 was selected to validate a transcript down-regulated in PAI-1$^{-/-}$ antral myofibroblasts relative to wild-type myofibroblasts (Table 4.2), and had in fact been flagged as absent in PAI-1$^{-/-}$ antral myofibroblasts using Affymetrix MAS5.0 summary algorithm. Secretogranin-2 is of particular interest as recent findings indicate that it is expressed by normal human gastric myofibroblasts and is a marker for a neuroendocrine-like phenotype of these cells, including the capacity for regulated secretion. This phenotype is lost in some gastric CAMs, particularly those from patients with advanced disease (Balabanova, 2012). In antral tissues of C57BL/6 and PAI-1$^{-/-}$ mice, secretogranin-2 was expressed by a sub-population of G-cells and by neurons, but not by myofibroblasts (Figures 4.5 & 4.6).

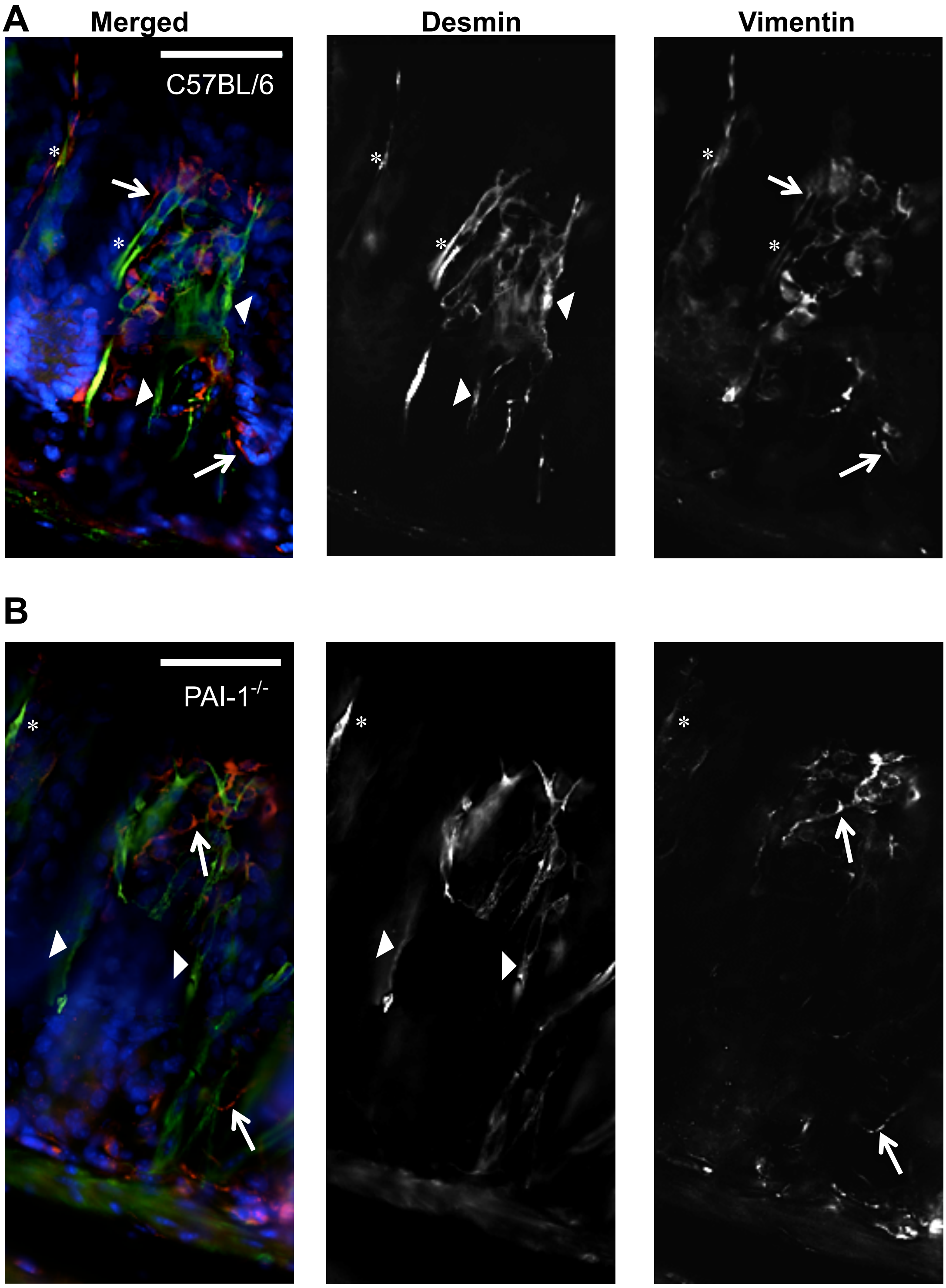


Figure 4.3. There are two distinct populations of desmin- and vimentin-expressing cells in mouse antral lamina propria. A: Desmin and vimentin immunopositive myofibroblast-like cells were located within the lamina propria of wild type antral tissue. There were few weakly co-localising cells (*). There were distinct populations of cells expressing either desmin (arrowheads) or vimentin (arrows). **B:** Similarly, two separate populations of desmin- and vimentin-expressing cells were seen in the lamina propria of PAI-1$^{-/-}$ antral tissue, with few weakly co-localising cells. Scale bars = 50µm.

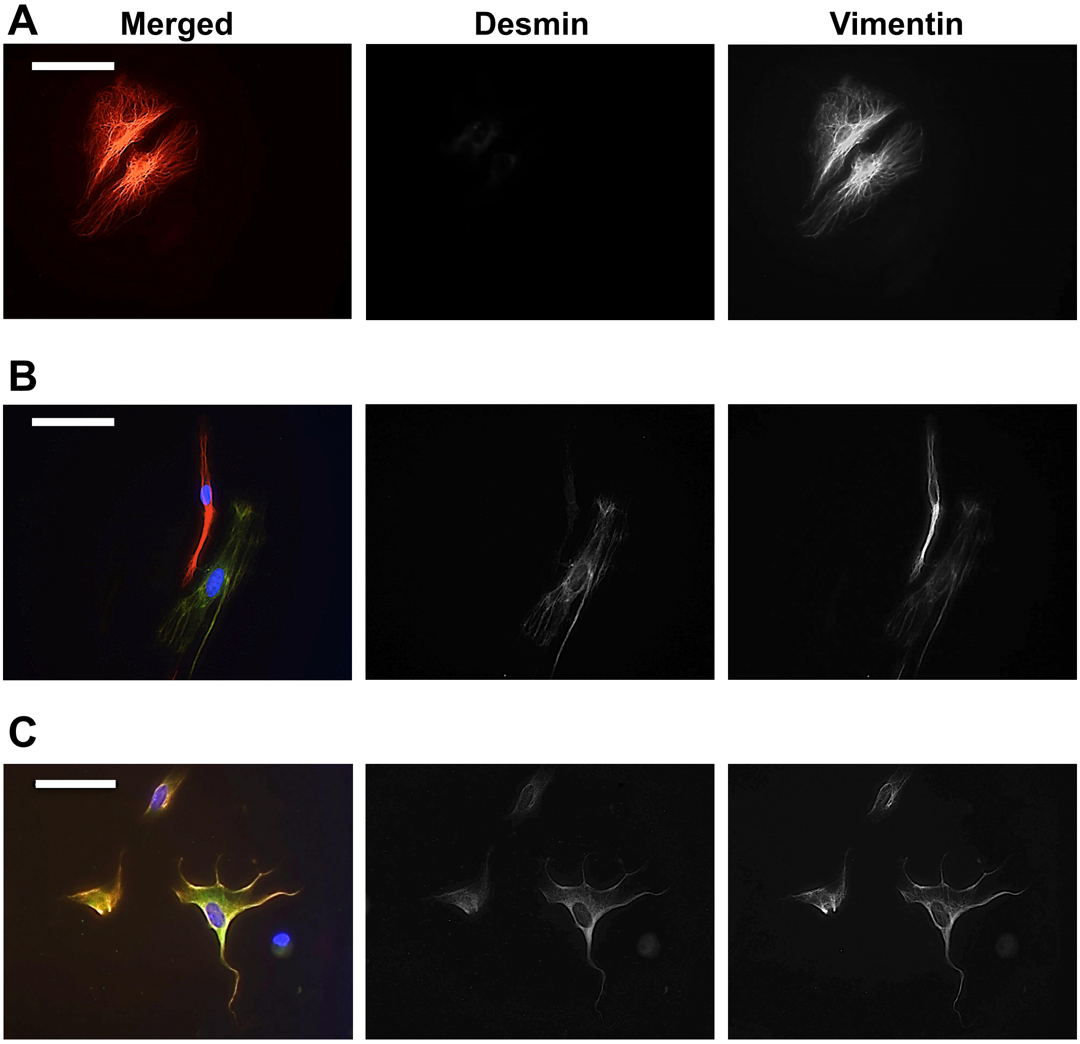


Figure 4.4. Dissociated antral myofibroblasts from PAI-1$^{-/-}$ mice express vimentin and desmin to varying degrees. Primary cultures of myofibroblasts isolated from antral tissue of PAI-1$^{-/-}$ mice consisted of a heterogeneous population of vimentin and desmin expressing cells. **A:** Cells strongly expressing vimentin but not expressing desmin. **B:** Mixed sample consisting of a cell strongly expressing desmin but very weakly expressing vimentin and a cell expressing vimentin but not desmin. **C:** A sample of cells co-expressing desmin and vimentin. Scale bars = 50μm.

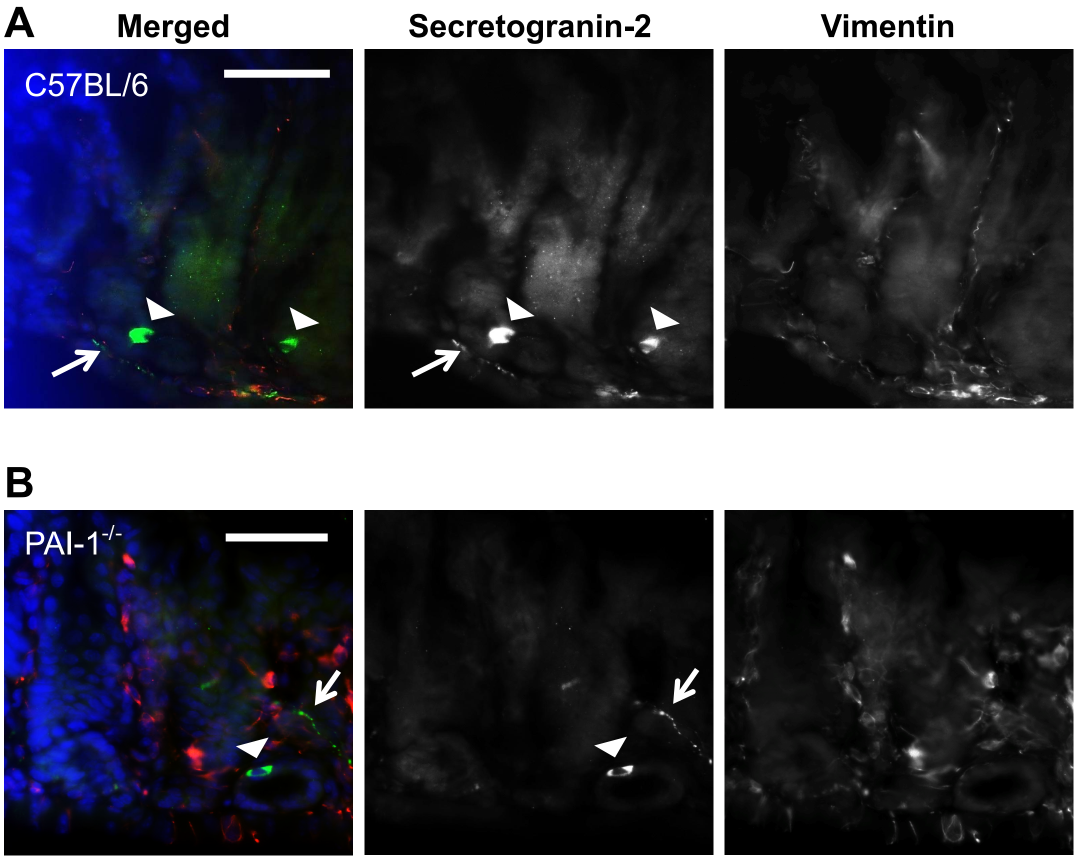


Figure 4.5. Secretogranin-2 is expressed by endocrine cells and neurons in antral mucosal tissue. A: Secretogranin-2 immunopositive cells consisted of endocrine cells (arrowheads) and neurons (arrows) in wild-type antral mucosa. Secretogranin-2 did not co-localise with vimentin-expressing cells. **B:** Similarly, secretogranin-2 was expressed by endocrine cells and neurons, but not by vimentin-expressing cells, in PAI-$1^{-/-}$ antral tissue. Scale bars = 50μm.

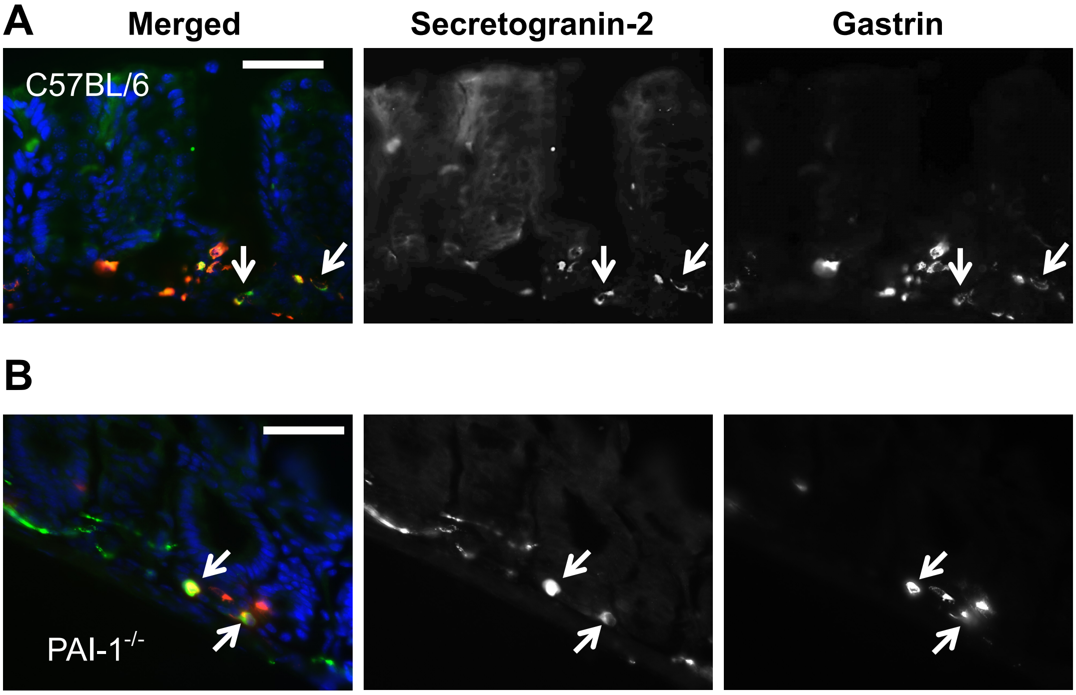


Figure 4.6. Secretogranin-2 is expressed by some G-cells. A: A sub-population of G-cells in wild type antrum expressed secretogranin-2, accounting for secretogranin-2 expressing antral endocrine cells (arrows). **B:** Similarly some, but not all, G-cells of PAI-1$^{-/-}$ antral tissue expressed secretogranin-2. All secretogranin-2 expressing endocrine cells were G-cells. Secretogranin-2 expressing neurons are also seen in this image. Scale bars = 50μm.

Unexpectedly, given the microarray findings, secretogranin-2 was expressed by dissociated PAI-$1^{-/-}$ antral myofibroblasts (Figure 4.7A).

Expression of DPP4 was investigated following its identification as an up-regulated transcript in PAI-$1^{-/-}$ antral myofibroblasts relative to wild-type myofibroblasts (Table 4.2), having been flagged as absent in C57BL/6 myofibroblasts. DPP4 was not detected by immunohistochemistry in any cell type in wild-type or PAI-$1^{-/-}$ antral mucosal sections (data not shown). However, DPP4 was expressed by dissociated PAI-$1^{-/-}$ antral myofibroblasts, where it was localised across the entire surface of cells (Figure 4.7B).

4.3.4 Expression of desmin, secretogranin-2 and dipeptidyl peptidase 4 by dissociated PAI-1-H/Kβ antral myofibroblasts is similar to PAI-$1^{-/-}$ myofibroblasts

The expression of desmin, secretogranin-2 and DPP4 was then studied in dissociated antral myofibroblasts from PAI-1-H/Kβ stomach, since these represented cells derived from a PAI-1 enriched microenvironment. Similar to PAI-$1^{-/-}$ myofibroblasts, isolated PAI-1-H/Kβ antral myofibroblasts existed as a heterogeneous population of vimentin and desmin expressing cells, with varying degrees of co-localisation of these two signals (Figure 4.8). Expression of secretogranin-2 and DPP4 by PAI-1-H/Kβ antral myofibroblasts was also similar to PAI-$1^{-/-}$ myofibroblasts (Figure 4.9).

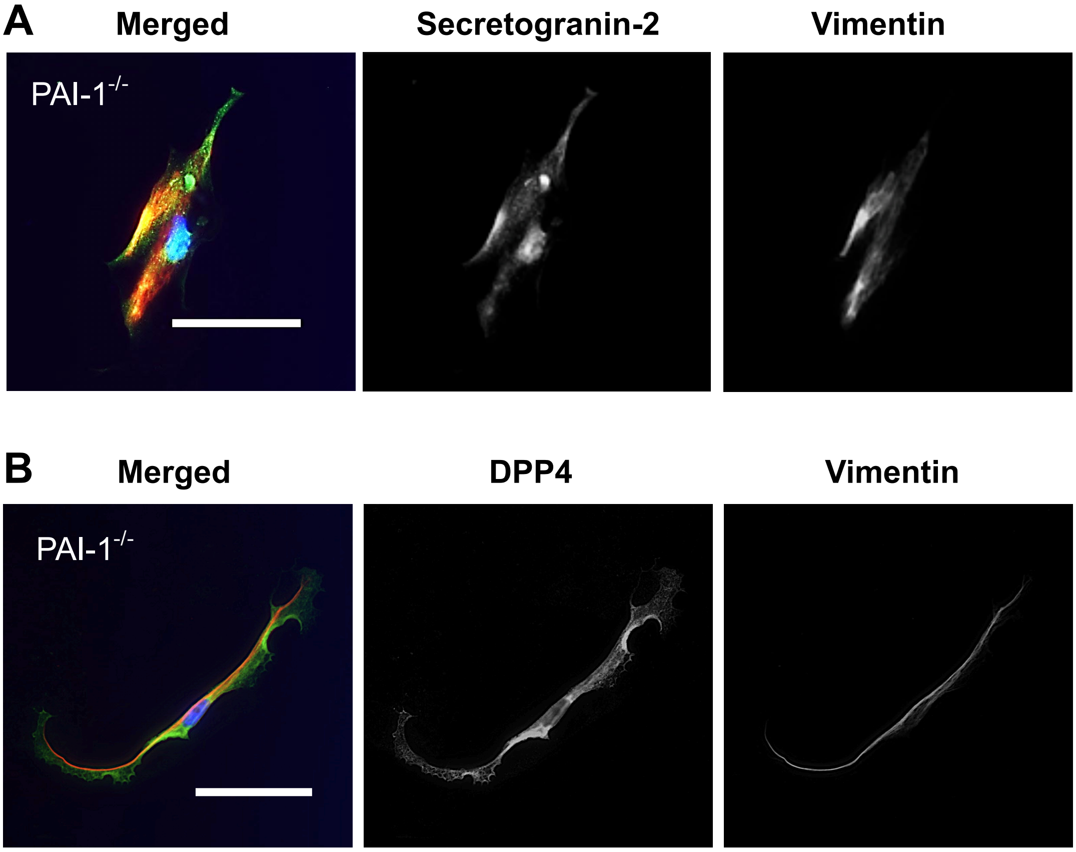


Figure 4.7. Dissociated antral myofibroblasts from PAI-1$^{-/-}$ mice express secretogranin-2 and dipeptidyl peptidase 4. A: Antral myofibroblasts from PAI-1$^{-/-}$ mice were secretogranin-2 immunopositive. Secretogranin-2 was seen as particulate staining within the cytoplasm, representing expression by secretory vesicles. **B:** A DPP4 expressing antral myofibroblast isolated from PAI-1$^{-/-}$ stomach. DPP4, a membrane bound protease, was expressed across the entire surface of cells. Scale bars = 50μm.

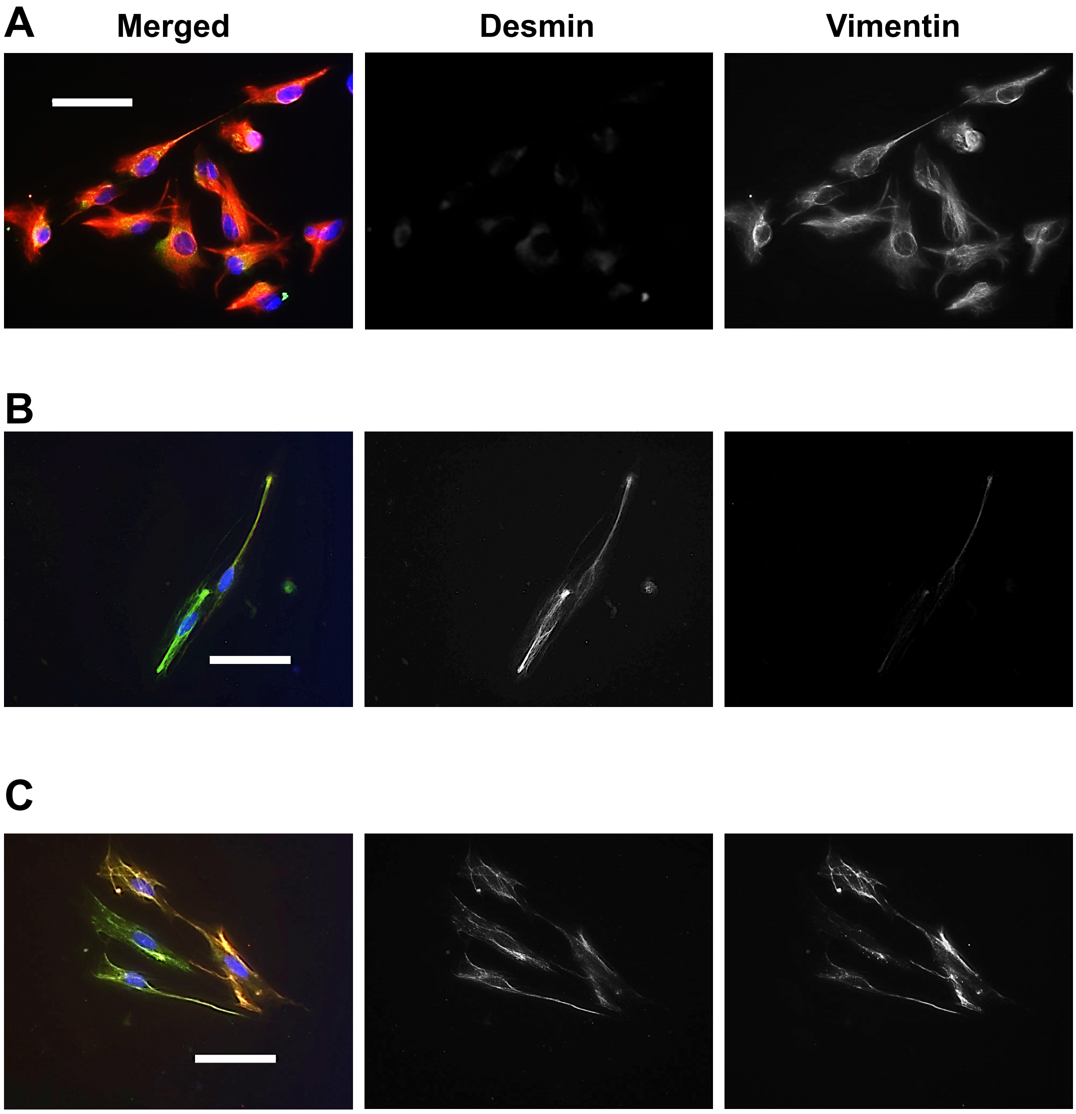


Figure 4.8. Dissociated antral myofibroblasts from PAI-1-H/Kβ mice consist of a mixed population of vimentin- and desmin-expressing cells. A: A cluster of cells expressing vimentin but not desmin. These cells had a more rounded morphology than other cells in the population. **B:** A spindle-shaped cell expressing desmin but only weakly expressing vimentin. **C:** A mixed sample of desmin-expressing cells, co-expressing vimentin to varying degrees. Scale bars = 50μm.

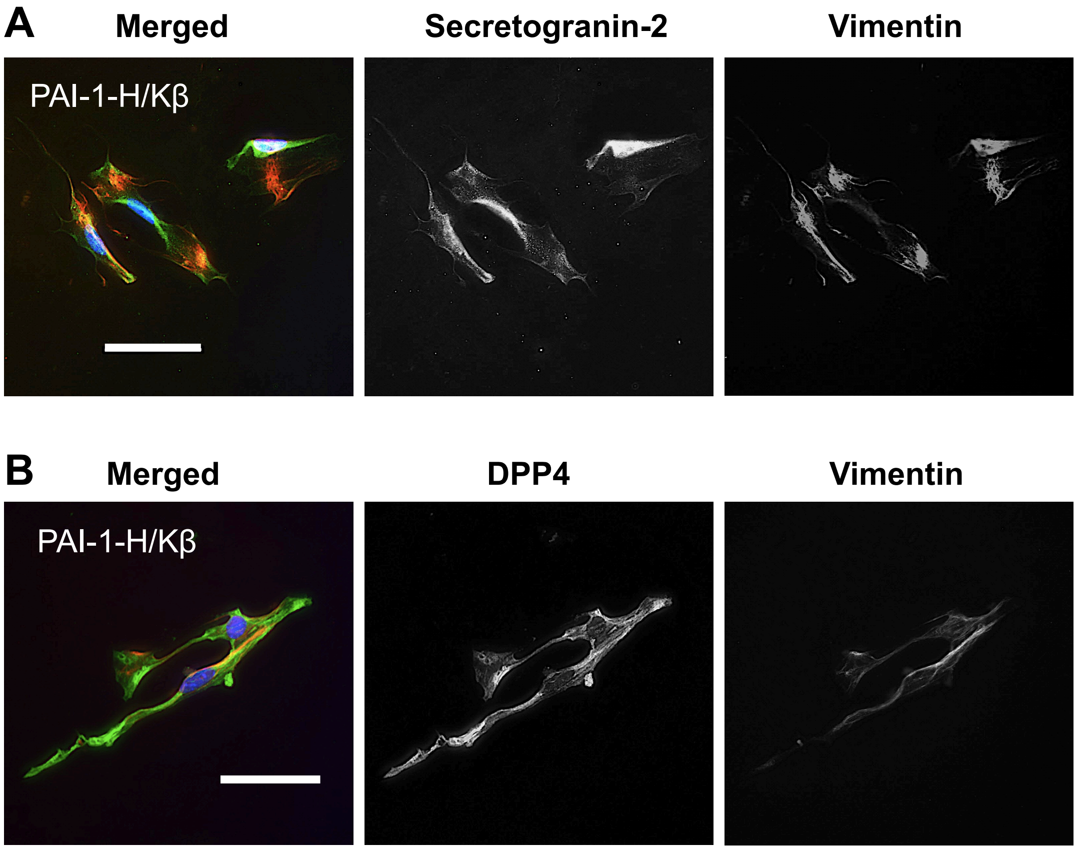


Figure 4.9. Dissociated antral myofibroblasts from PAI-1-H/Kβ mice express secretogranin-2 and dipeptidyl peptidase 4. A: Secretogranin-2 immunopositive PAI-1-H/Kβ antral myofibroblasts. **B:** DPP4 was expressed across the surface of antral myofibroblasts isolated from PAI-1-H/Kβ stomach. Scale bars = 50μm.

4.3.5 Treatment of human antral myofibroblasts with exogenous PAI-1 does not affect dipeptidyl peptidase 4 activity

Immunocytochemical analysis determined that cultured human antral myofibroblasts, characterised on the basis of positive α-SMA and vimentin expression, did not express desmin (Figure 4.10). DPP4 was expressed across the entire surface of all human antral myofibroblasts (Figure 4.11). The hypothesis that PAI-1 negatively regulates expression of DPP4 by antral myofibroblasts was tested by comparing the DPP4 activity of PAI-1 treated and untreated cultured human antral myofibroblasts, using a colorimetric enzymatic assay. The chromogenic molecule pNA was generated via cleavage of Gly-Pro-pNA by DPP4 (Nagatsu *et al.*, 1976). The relationship between the number of myofibroblasts plated and DPP4 activity was linear (Figure 4.12A).

DPP4 activity of myofibroblasts plated for 48 and 72 hours was constant but increased after 96 hours of plating (Figure 4.12A) There was no significant difference in DPP4 activity of myofibroblasts treated with 50nM PAI-1 for 3, 5 or 7 days compared to untreated myofibroblasts (Figure 4.12B).

4.3.6 DPP4 activity of PAI-1-H/Kβ mouse plasma is elevated compared to wild-type and PAI-1$^{-/-}$ plasma

A fluorimetric enzymatic assay based on the generation of AMC via cleavage of Gly-Pro-AMC by DPP4 was used to compare DPP4 activity of plasma from C57BL/6, PAI-1$^{-/-}$ and PAI-1-H/Kβ mice (Kato *et al.*, 1978). Fluorescence developed consistently, in a linear manner, over a 30 min assay period (Figure 4.13A). DPP4 activity of plasma from PAI-1-H/Kβ mice was significantly elevated

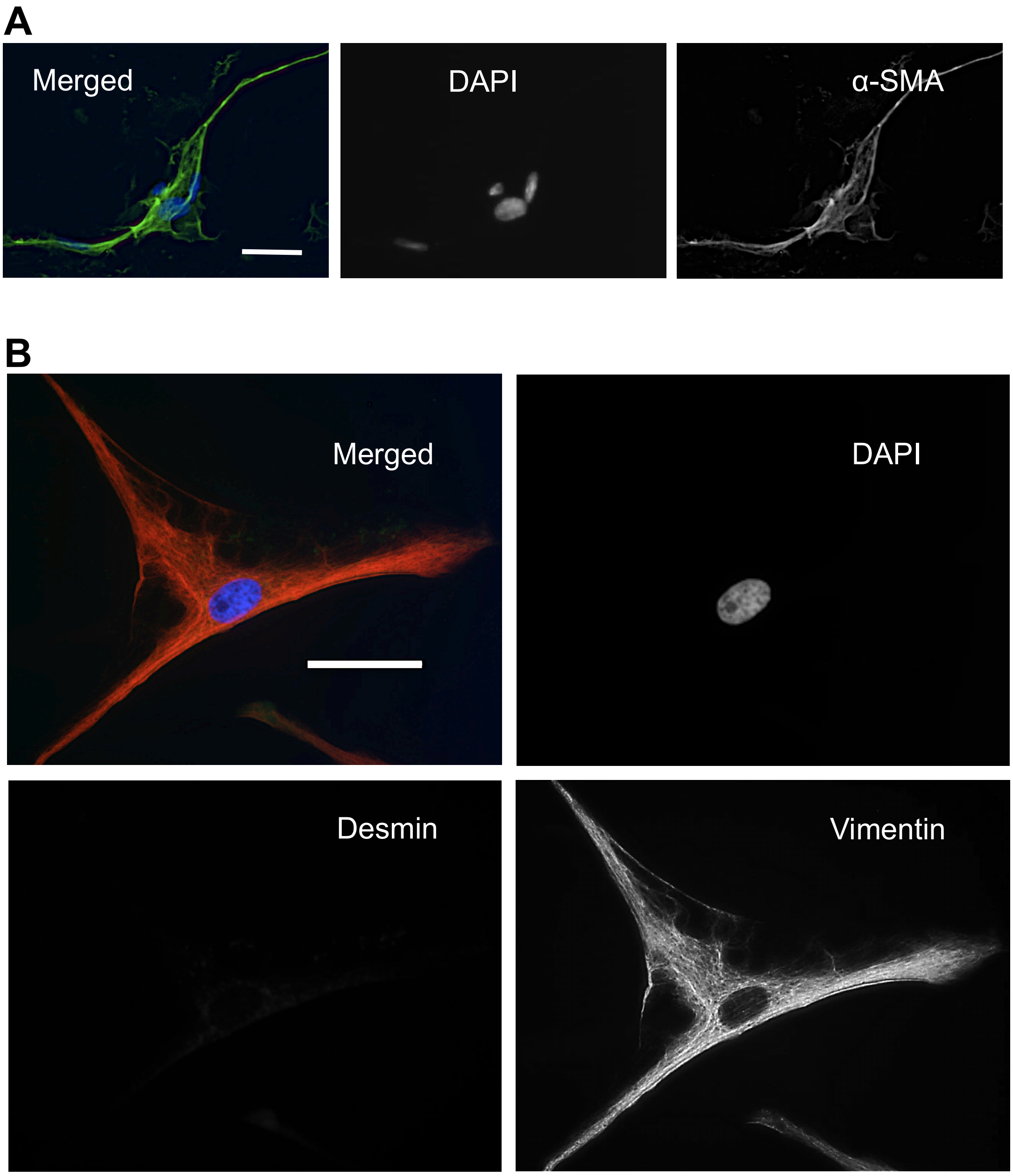


Figure 4.10. Cultured human gastric myofibroblasts express α-smooth muscle actin and vimentin, but not desmin. A: Myofibroblasts that were isolated from a human gastric biopsy were α-SMA immunopositive. **B:** These myofibroblasts also strongly expressed vimentin, but did not express desmin. Scale bars = 50μm.

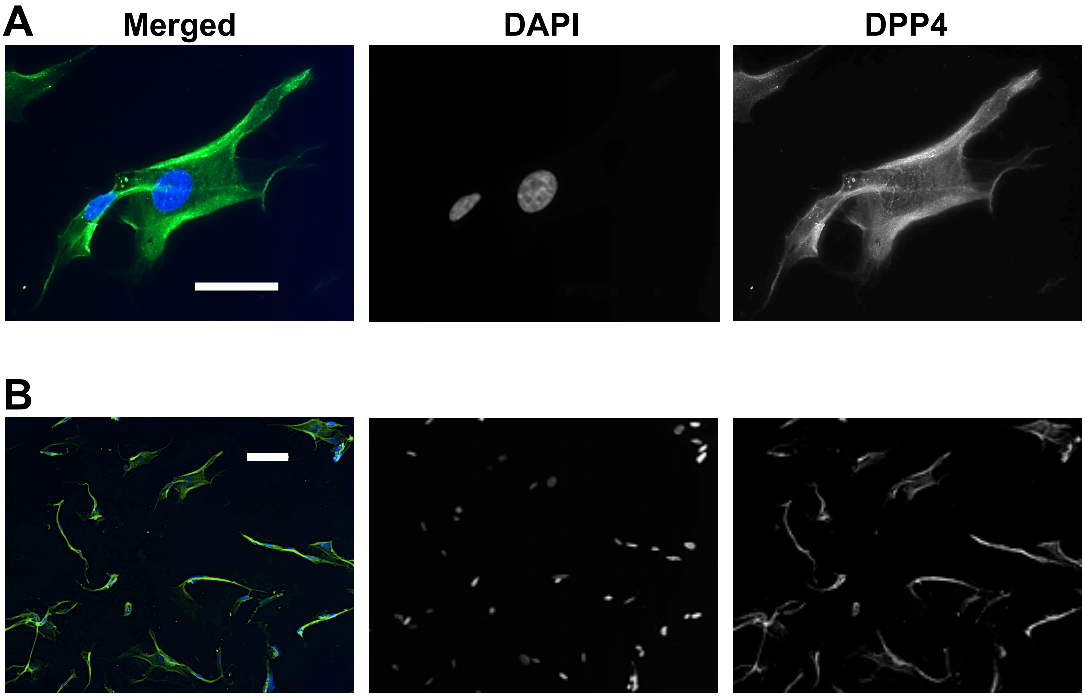


Figure 4.11. Cultured human gastric myofibroblasts express DPP4 across the entire cell surface. A: High power magnification (x40) of cultured human gastric myofibroblasts demonstrated that these cells expressed DPP4 on the cell surface. **B:** Low power magnification (x10) imaging illustrated that the entire population of cells expressed DPP4. Scale bars = 50µm (high power) and 200µm (low power).

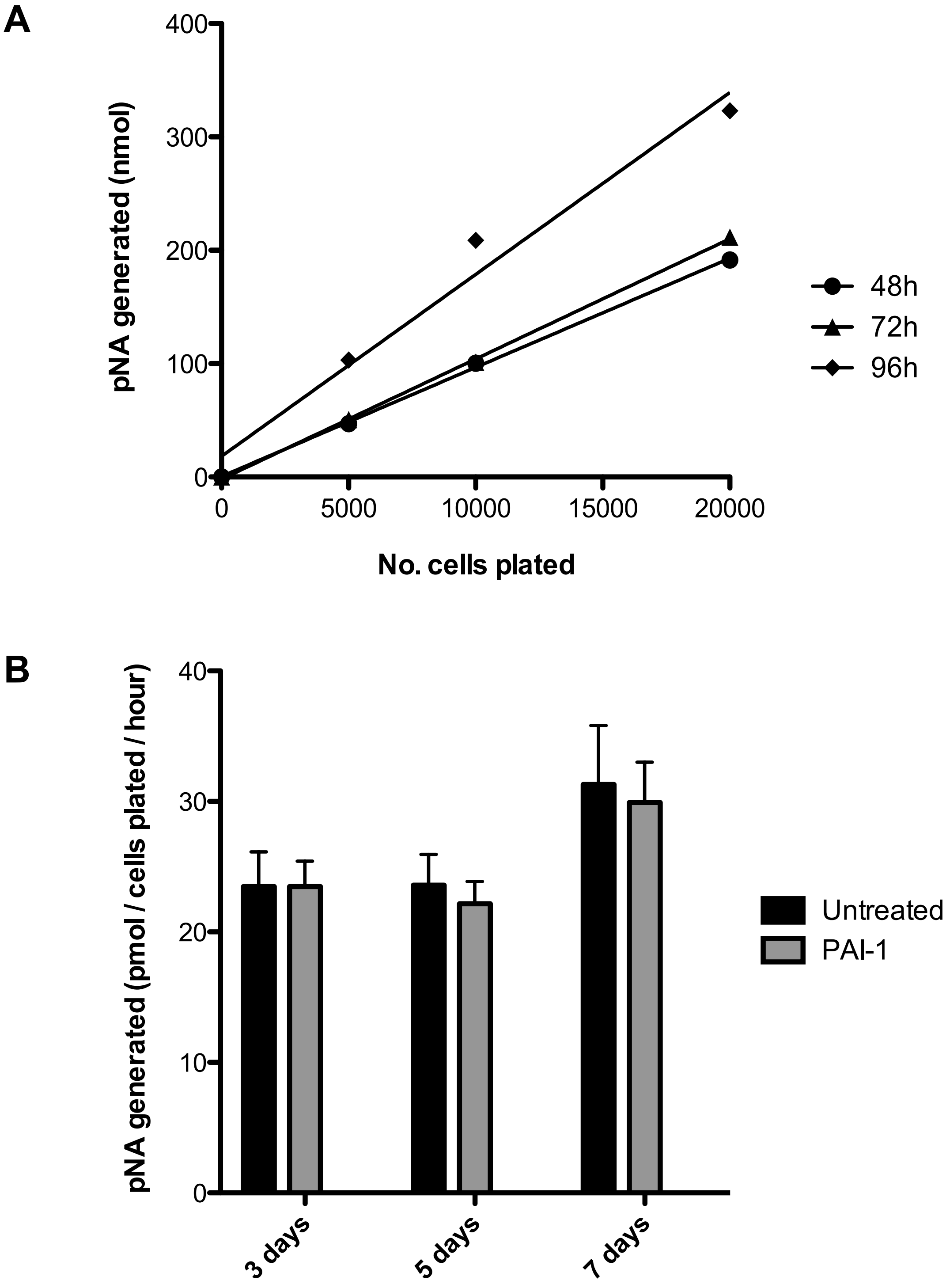


Figure 4.12. DPP4 activity of cultured human myofibroblasts treated with 50nM PAI-1 is similar to untreated cells. A: There was a linear relationship between the number of myofibroblasts plated and DPP4 activity of the cultured cells. The cells were assayed 48, 72 or 96 hours after plating. There was an increase in DPP4 activity of myofibroblast cultures plated for 96 hours, compared to 48 and 72 hours. **B:** 10000 myofibroblasts were plated and treated with 50nM PAI-1 24 hours later. DPP4 activity was assayed 3, 5 and 7 days after treatment. PAI-1 treatment had no significant effect on DPP4 activity at any of these time points. Data represent the mean of at least 3 individual experiments ± SEM.

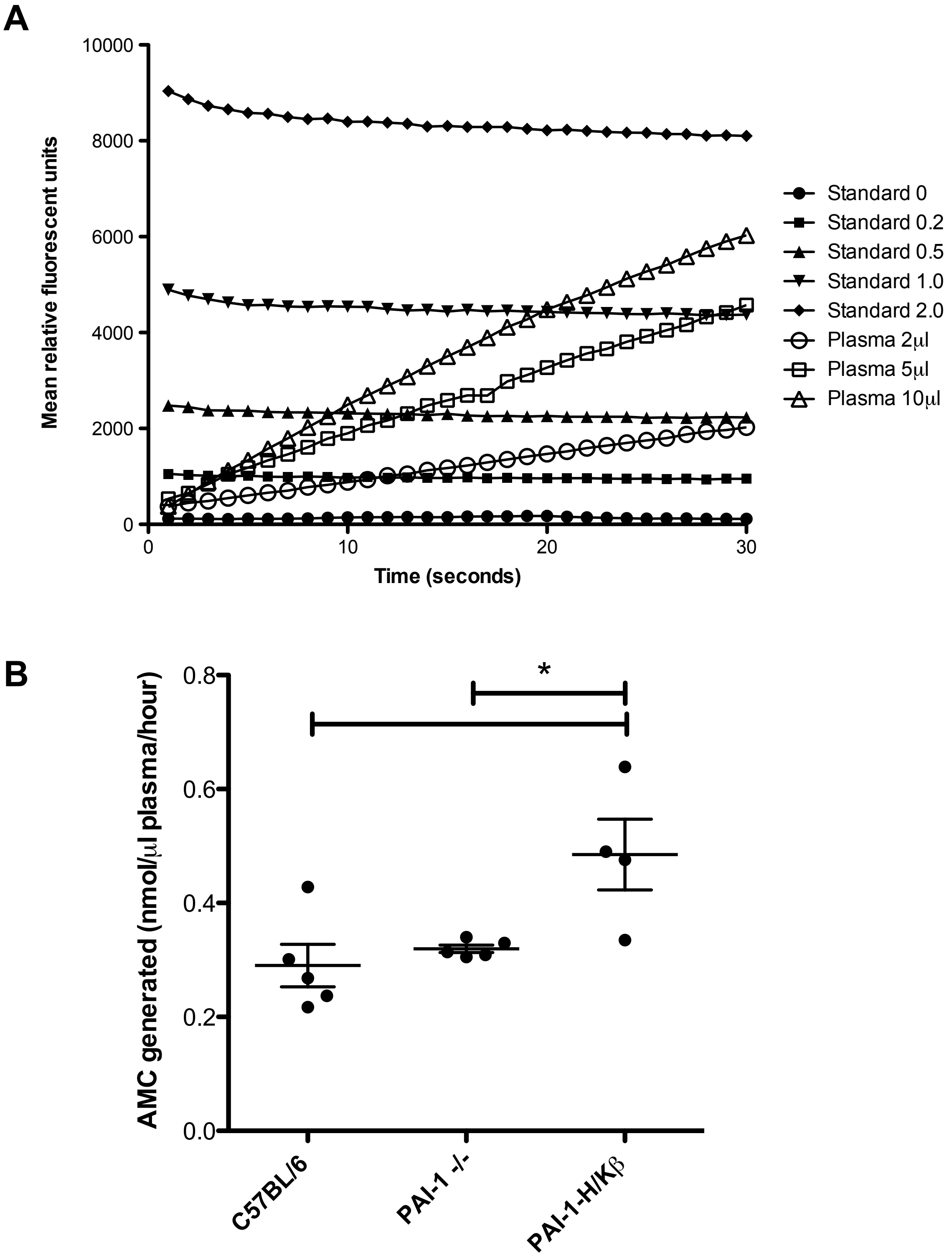


Figure 4.13. DPP4 activity of plasma from PAI-1-H/Kβ mice is elevated compared to wild type and PAI-1$^{-/-}$ plasma. A: DPP4 activity of 2, 5 and 10μl mouse plasma remained constant up to 30 min assay duration, developing fluorescence via the generation of AMC in a linear manner. **B:** DPP4 activity of 5μl plasma from C57BL/6, PAI-1$^{-/-}$ and PAI-1-H/Kβ mice was assayed over a 20 min period. DPP4 activity of PAI-1-H/Kβ plasma was significantly higher than C57BL/6 and PAI-1$^{-/-}$ plasma. Data for individual animals are shown in addition to mean ± SEM. * $P < 0.05$ (one-way ANOVA).

compared to both wild-type and PAI-1$^{-/-}$ plasma (Figure 4.13B). There was no difference in DPP4 activity of C57BL/6 and PAI-1$^{-/-}$ plasma.

4.4 Discussion

It is known that gastric epithelial and stromal cells express PAI-1, and that expression of PAI-1 is elevated at the site of gastroduodenal ulcers, in chronic gastritis, in hypergastrinaemia and in gastric cancer (Tanaka *et al.*, 1991; Nakamura *et al.*, 1992; Wodziński *et al.*, 1993; Nekarda *et al.*, 1994; Farinati *et al.*, 1996; Herszenyi *et al.*, 1997; Plebani *et al.*, 1997; Kaneko *et al.*, 2003; Kenny *et al.*, 2008; Keates *et al.*, 2008; Ikeda *et al.*, 2009; Norsett *et al.*, 2011). The influence of PAI-1 on gastric myofibroblast gene expression and subsequently on myofibroblast-epithelial interactions had not previously been investigated. The aim of the work described in this chapter was to identify potential myofibroblast-derived modulators of the gastric mucosal microenvironment, expression of which is influenced by gastric PAI-1 expression. Global gene expression profiles of wild-type and PAI-1$^{-/-}$ antral myofibroblasts were compared, and differentially expressed transcripts of secreted proteins were identified to uncover potential PAI-1-regulated modulators of the gastric mucosal microenvironment. Microarray analysis indicated that global gene expression profiles of C57BL/6 and PAI-1$^{-/-}$ myofibroblasts were distinct from each other, with a large number of differentially expressed transcripts.

The MAS5.0 summary algorithm was used to normalise raw signal intensity data generated by the microarray. This is Affymetrix's proprietary pre-processing algorithm, adjusting signal intensity values to remove noise due to inherent technical variations in the processing of microarray chips, including background subtraction and chip-wise normalisation (Hubbell *et al.*, 2002). Since the microarrays were processed simultaneously, normalisation of batch effects was not an issue. Unlike other pre-processing algorithms, the MAS5.0 summary algorithm uses mismatch probes to generate calls for each probe set, allowing exclusion of non-expressed transcripts from the analysis. To further eliminate noise from the analysis, undetected transcripts were filtered by selecting only those entities flagged as present in all samples of C57BL/6 or PAI-1$^{-/-}$ myofibroblasts. An arbitrary fold-change cut-off value of ⩾ 3 or ⩽ -3 was selected, to define differentially expressed transcripts. This approach does not take into account the fact that signal intensity values of transcripts that are expressed at low levels will be subject to much greater error, increasing the chance of false-positive detection at any arbitrary fold-change cut-off value, whilst the detection rate of highly expressed transcripts that are differentially expressed is insufficient. A better method might involve defining an appropriate fold-change cut-off value for each transcript, as a function of its absolute expression level relative to other transcripts in the same range of expression level (Mutch *et al.*, 2002). However, it is important to consider that many potential false-positives are eliminated from the analysis by using the MAS5.0 summary algorithm, combining detection calls and fold-change cut-off values to identify differentially expressed transcripts, making this both a selective and sensitive algorithm (Pepper *et al.*, 2007).

Gastric myofibroblasts cultured for gene expression analysis had been characterised on the basis of positive α-SMA and vimentin expression by immunocytochemistry. The microarray data confirmed expression of the corresponding transcripts. Vimentin and α-SMA have been used previously to characterise mouse and human gastric myofibroblasts by immunohistochemistry (Wu *et al.*, 1999; Fuyuhiro *et al.*, 2011; Holmberg *et al.*, 2012). Expression of these proteins marks cells as having characteristics of both fibroblasts and smooth muscle cells: the classical definition of the myofibroblast (Majno *et al.*, 1971). A more rigorous definition of the myofibroblast has since been argued based on expression of α-SMA and at least three other markers, which may include γ-SMA, vimentin, desmin, endosialin, palladin 4Ig, prolyl-4 hydroxylase, podoplanin, cadherin-11 or negative markers such as epithelial cytokeratins, smoothelin, CD14, CD31 and CD34 (De Wever *et al.*, 2008). The microarray data revealed that cultured gastric myofibroblasts from C57BL/6 and PAI-$1^{-/-}$ mice met these criteria.

Immunofluorescence was used to characterise expression of desmin by antral myofibroblasts on the basis that PAI-$1^{-/-}$ myofibroblasts, but not C57BL/6 myofibroblasts, had been shown to express desmin mRNA. Unexpectedly, this led to the discovery of a mixed population of desmin- and vimentin-expressing myofibroblast-like cells in both C57BL/6 and PAI-$1^{-/-}$ antral sections. Furthermore, acutely cultured antral PAI-$1^{-/-}$ and PAI-1-H/Kβ myofibroblasts were shown to variably express desmin by immunocytochemistry. This is consistent with a previous study reporting that a mixed population of desmin-positive and desmin-negative myofibroblasts were identified, migrating out of denuded gastric mucosa (Wu *et al.*, 1999). This is the first time that a heterogeneous myofibroblast population, in terms of the expression of desmin, has been identified *in situ* within the lamina propria of gastric mucosa.

Previous studies report conflicting results regarding the expression of desmin by gastrointestinal myofibroblasts. In a previous study undertaken in this laboratory, a total of 30 human myofibroblast lines were examined, including gastric CAMs, ATMs and NTMs, all of which were α-SMA and vimentin-positive but desmin-negative (Holmberg *et al.*, 2012). The results reported in this chapter are consistent with these observations. In C57BL/6 mice, desmin-negative myofibroblasts appeared within the corpus mucosa and submucosa following cryo-ulceration (Ashurst *et al.* 2008). Normal colonic pericryptal myofibroblasts have been identified as being desmin-negative, whereas desmin-expressing myofibroblasts appeared focally in adenomatous and hyperplastic polyps (Adegboyega *et al.*, 2002). Conversely, desmin-positive myofibroblasts have been identified along the normal canine gastrointestinal tract, yet myofibroblasts of canine gastrointestinal tumour stroma were desmin-negative, except those adjacent to the muscularis mucosae (Mukaratirwa *et al.*, 2003). This led the authors to propose that myofibroblasts in canine tumour stoma derive primarily from fibroblasts, with few originating from smooth muscle cells of the muscularis mucosae. The desmin-positive myofibroblasts observed in mouse antral sections and in culture may derive from smooth muscle cells of the muscularis mucosae, vasculature or submucosal muscle layers. Further characterisation of these cells in terms of expression of smooth muscle markers, such as smooth muscle myosin, would help to elucidate this point. Furthermore, it has been suggested that ultrastructural examination may better identify features of ‘true myofibroblasts’, and distinguish cells that are more characteristic of smooth muscle cells that might otherwise be identified as myofibroblasts by immunofluorescence (Eyden, 2008).

It is becoming increasingly apparent that myofibroblasts exist as a heterogeneous population of cells derived from and with features of multiple precursors, including fibroblasts, mesenchymal stem cells, smooth muscle cells, pericytes, epithelial cells, myoepithelial cells, endothelial cells, fibrocytes, adipocytes and stellate cells (Powell *et al.*, 2005; De Wever *et al.*, 2008; Powell *et al.*, 2011). The classical characterisation of myofibroblasts based on expression of α-SMA and vimentin is therefore seen to fail to adequately describe the complexity of myofibroblast populations in normal and pathophysiological tissues. This formed the basis of the proposal of more stringent criteria to distinguish myofibroblasts from other myofibroblast-like cells, such as CAFs and activated fibroblasts (De Wever *et al.*, 2008; Eyden, 2008). The existence of a heterogeneous myofibroblast population in mouse antrum is supported by the observation that transcripts of several stromal cell markers were differentially expressed by wild-type and PAI-1$^{-/-}$ cultured antral myofibroblasts, including α-SMA and desmin, the expression of which was significantly elevated by PAI-1$^{-/-}$ myofibroblasts. This may be indicative of a more smooth muscle cell-like phenotype of this population of cells, which were selectively cultured from PAI-1$^{-/-}$ antral tissue.

Myofibroblasts adapt to their microenvironment, allowing them to participate in tissue remodelling by taking on a contractile and secretory phenotype in granulation tissue and supporting carcinogenesis in tumour stroma. Given the microenvironment-dependent phenotypic plasticity of myofibroblasts, adaptations to culture conditions would not be surprising. For example, it has recently been reported that the proportion of α-SMA-expressing myofibroblasts decreased with serial passage of gastric fibroblast/myofibroblast cultures, and the myofibroblast population could be recovered by supplementation with conditioned medium from scirrhous gastric carcinoma cells, via TGF-β (Fuyuhiro *et al.*, 2011). Myofibroblasts used for microarray studies had been passaged 3 or 4 times. Therefore, it seems possible that the expression of secretogranin-2 and DPP4 observed in cultured antral myofibroblasts, but not by myofibroblasts in antral sections, provides a further example of adaptation to culture conditions. Furthermore, adaptation to culture conditions is likely to occur rapidly, since acutely cultured myofibroblasts used for microarray validations also expressed secretogranin-2 and DPP4.

The phenomenon of cellular adaptation to culture conditions is well documented. Studies examining global gene expression profiles of cultured cells and corresponding intact tissues have shown that although primary cell cultures are more similar to original tissues than immortalised cell lines, significant changes in gene expression still occur (Dairkee *et al.*, 2004; Sandberg & Ernberg, 2005). The limitations of two-dimensional cultures to recapitulate the complexity of interactions between stromal cells, parenchymal cells and the ECM necessarily leads to genetic, transcriptional and post-translational adaptations (Ross *et al.*, 2000; Roschke *et al.*, 2003; Irish *et al.*, 2004; Birgersdotter *et al.*, 2005). The question arises as to whether cultured mouse antral myofibroblasts develop a neuroendocrine-like phenotype, marked by expression of secretogranin-2, and regulated secretion of certain proteins, such as DPP4. The neuroendocrine-like phenotype of human myofibroblasts has been well-characterised, based on expression of secretogranin-2 and the capacity for regulated secretion, which is lost in some gastric cancer patients with poor survival (Balabanova, 2012).

To confirm that secretogranin-2 expression by gastric myofibroblasts is induced in culture, transgenic mice could be generated with the green fluorescent protein (GFP) reporter gene coupled to the secretogranin-2 promoter. Based on this hypothesis, myofibroblasts in *ex vivo* antral tissue would express minimal levels of

GFP, whilst expression by cultured antral myofibroblasts would be significantly elevated. Furthermore, adaptation of gastric myofibroblasts to a neuroendocrine-like phenotype in culture may be a physiologically relevant model of the phenotypic adaptations during wound healing and inflammation. To explore this hypothesis, wounded and chronically inflamed tissues from GFP-secretogranin-2 reporter mice could be examined *ex vivo*, to determine whether myofibroblasts express secretogranin-2 in these microenvironments.

Certain limitations exist in the approach that was taken to validate the microarray data by immunocytochemistry, in that myofibroblasts used for the validations were freshly dissociated and acutely cultured, whilst those used to prepare material for the microarrays had been passaged 3 or 4 times. As discussed above, it is now clear that mouse myofibroblasts exist as a heterogeneous population of desmin-positive and desmin-negative cells, quite distinct from the situation in humans (Holmberg *et al.*, 2012). This raises the possibility of subclone selection in culture. Together with the phenotypic plasticity of cultured myofibroblasts, this highlights the need to use myofibroblasts with similar passage histories for gene or protein expression analysis and subsequent validation studies, and also to characterise myofibroblasts on the basis of expression of multiple markers, including α-SMA, vimentin and desmin, to confirm that these populations are equivalent and the validations are therefore valid.

In terms of potential modulators of epithelial cell proliferation, DPP4 was of particular interest. DPP4 cleaves IGF-I, generating a truncated form that has impaired IGF-I receptor-activating potential and a greater affinity for IGFBP-3 (Lin *et al.*, 2010). Microarray data revealed that DPP4 mRNA was expressed by PAI-1$^{-/-}$

cultured antral myofibroblasts but not C57BL/6 myofibroblasts, leading to the hypothesis that DPP4 is down-regulated by PAI-1, increasing epithelial cell proliferation by increasing the bioavailability of active IGF-I. To determine the effect of increased PAI-1 concentrations on myofibroblast DPP4 activity, cultured human myofibroblasts were treated with exogenous PAI-1 and DPP4 activity was assayed at various time-points following treatment. PAI-1 did not significantly alter DPP4 activity 3, 5 or 7 days after treatment with PAI-1. Furthermore, PAI-1-H/Kβ mouse plasma had elevated DPP4 activity compared to wild-type and PAI-$1^{-/-}$ plasma, which provides no support for the hypothesis that PAI-1 negatively regulates DPP4. However, plasma DPP4 activity may not reflect the total expression of DPP4 by cells, given that this only represents the bioavailability of the truncated soluble DPP4 isoform, and does not provide information about cell-surface bound DPP4.

Future investigations might be directed towards identifying other differentially expressed transcripts in the microarray data that could influence epithelial cell proliferation. One striking candidate is pleiotrophin, the most differentially expressed transcript by C57BL/6 myofibroblasts compared to PAI-$1^{-/-}$ myofibroblasts. Pleiotrophin mRNA had in fact been flagged as absent in PAI-$1^{-/-}$ myofibroblasts, indicating a potential positive regulatory role for PAI-1. Pleiotrophin is a developmental growth factor, known to promote neurite outgrowth, and is mitogenic in a range of other tissues, stimulating fibroblasts, endothelial cells and epithelial cells (Rauvala, 1989; Li *et al.*, 1990; Courty *et al.*, 1991; Wellstein *et al.*, 1992; Fang *et al.*, 1992; Laaroubi *et al.*, 1994; Choudhuri *et al.*, 1997). It is of interest that the microarray data indicated that midkine, a protein related to pleiotrophin with 45% sequence homology, was also significantly differentially expressed by C57BL/6 antral myofibroblasts compared to PAI-1$^{-/-}$ myofibroblasts, being flagged as absent in PAI-1$^{-/-}$ myofibroblasts. Pilot studies were conducted to investigate the effect of IP administration of pleiotrophin on gastric epithelial cell proliferation *in vivo*, using an EdU based assay, but these did not produce any positive data. It would be useful to characterise the relative expression of pleiotrophin and midkine in wild-type and PAI-1$^{-/-}$ gastric tissues, using Western blotting and quantitative polymerase chain reaction (qPCR), and assays to investigate the mitogenic potential of pleiotrophin in the gastric mucosa *in vivo* require more optimisation. Similar approaches may also be used to investigate the functional significance of other differentially expressed transcripts in the microarray data.

The data presented in this chapter provides novel insights into the characteristics of myofibroblasts resident in mouse antral tissues. Two highly heterogeneous populations of myofibroblasts can be found in mouse gastric mucosa, with distinct global gene expression profiles. One of these expresses markers suggestive of a more smooth-muscle cell-like differentiation. Furthermore, the data raise the possibility that myofibroblasts adapt to culture conditions by developing a neuroendocrine-like phenotype, which may be relevant to the compromised gastric mucosal microenvironment, such as in *Helicobacter* infection and NSAID-induced injury. Based on these observations indicating the complexity of gastric myofibroblast phenotypes, a major outcome of these studies was clarification of how studies on gastric myofibroblasts need to be designed and validated, to ensure that equivalent populations are investigated.

4.5 Conclusions

1. Cultured antral mucosal myofibroblasts from wild-type and PAI-1$^{-/-}$ mice have distinct global gene expression profiles.
2. Mouse antral mucosal myofibroblasts exist as a heterogeneous population of vimentin- and desmin-expressing cells, *in situ* and in acutely dissociated cell culture.
3. Mouse antral mucosal myofibroblasts may develop a neuroendocrine-like phenotype in culture conditions.
4. Exogenous PAI-1 does not influence the DPP4 activity of cultured human antral myofibroblasts.
5. DPP4 activity of PAI-1-H/Kβ mouse plasma is increased, compared to wild-type and PAI-1$^{-/-}$ plasmas.

CHAPTER 5

THE ROLE OF PAI-1 IN HISTOPATHOLOGICAL RESPONSES TO CHRONIC *H. FELIS* INFECTION

5.1 Introduction

PAI-1 influences inflammatory responses to, and outcomes of, various infections. On the one hand, PAI-1 expression correlates with worse outcomes in sepsis, and carriers of the 4G allele of PAI-1 polymorphism, associated with increased expression of PAI-1, have a poorer prognosis following pneumonia-induced sepsis (Mesters *et al.*, 1996; Tani *et al.*, 2001; Raaphorst et a*l.*, 2001; Madach *et al.*, 2010). However, expression of PAI-1 associated with systemic *Yersinia enterocolitica* infection, *Klebsiella pneumoniae* infection, *Haemophilus influenzae* infection and pneumonia caused by *Pseudomonas areuginosa*, is associated with effective host immune responses, bacterial clearance and improved outcomes, whilst PAI-1 does not effect host responses to *Streptococcus pneumoniae* infection (Rijneveld *et al.*, 2003; Renckens *et al.*, 2007; Lim *et al.*, 2011; Wang et al., 2013). Gastric expression of PAI-1 is elevated in response to *Helicobacter* infection (Herszenyi *et* al, 1997; Kenny *et al.*, 2008; Keates *et al.*, 2008; Ikeda *et al.*, 2009). The functional significance of increased expression of PAI-1 in the stomach during *Helicobacter* infection remains to be determined.

Previous work in this laboratory has shown that gastric PAI-1 acts as an orexigenic agent, attenuating CCK-evoked gut-brain satiety signals (Kenny *et al.*, 2013a). Given that gastric PAI-1 expression is increased during *Helicobacter* infection, it was proposed that PAI-1 counteracts inflammation-induced anorexigenic signals in response to *Helicobacter* infection, maintaining energy balance in the face of chronic gastritis (Kenny *et al.*, 2013a). The work in this chapter explores the role of PAI-1 in histopathological responses to *H. felis* infection in mice, and in particular the possibility that PAI-1 also maintains gastric mucosal homeostasis.

5.1.1 Aims and objectives

The aim of this chapter was to determine the gastric histopathological responses to *H. felis* infection in PAI-1$^{-/-}$ and PAI-1-H/Kβ mice compared to C57BL/6 mice, 48 weeks after initial infection. The specific objectives were to:

1. determine antral colonisation by *H. felis* and antral morphology following chronic *H. felis* infection of PAI-1$^{-/-}$ and PAI-1-H/Kβ mice compared to wild-type mice;
2. determine the degree of *H. felis*-induced corpus inflammation of PAI-1$^{-/-}$ and PAI-1-H/Kβ mice compared to wild-type mice;
3. determine changes in corpus mucosal myofibroblast abundance following chronic *H. felis* infection of PAI-1$^{-/-}$ and PAI-1-H/Kβ mice compared to wild-type mice;
4. determine corpus mucosal thickness, foveolar hyperplasia, oxyntic gland atrophy and epithelial defects, in response to chronic *H. felis* infection of PAI-1$^{-/-}$ and PAI-1-H/Kβ mice compared to wild-type mice.

5.2 Methods

5.2.1 *H. felis* infection

Male and female C57BL/6, PAI-1$^{-/-}$ and PAI-1-H/Kβ mice aged 6-8 weeks were infected with a suspension of *H. felis* in TBS broth, as described in section 2.2.3. Animals were humanely killed after 48 weeks alongside age-matched uninfected mice of each strain, and gastric tissues were harvested for histological analysis. Infected and uninfected treatment groups consisted of 6 – 9 animals of each strain.

5.2.2 Histology

Stomachs were prepared for either frozen sections or paraffin-embedded sections, as described in sections 2.3 and 2.4 respectively. Paraffin-embedded sections were stained with H&E, Giemsa stain or were vimentin immunostained whilst frozen sections were stained with Texas Red-conjugated-secondary antibody directed against vimentin primary antibody, to identify mucosal myofibroblasts.

5.2.3 Histopathological scoring

Colonisation of antral glands with Giemsa-positive *H. felis* was quantified on a scale of 0 – 4, as described in section 2.12. Similarly, corpus mucosal myofibroblast abundance was graded from 0 – 4, based on the number of vimentin-immunopositive cells, as described in section 2.12. Antral and corpus mucosal thickness was determined using AxioVision 4.5 software, as described in section 2.12. Sections stained with H&E were used to characterise tissue morphology and corpus histopathological features were quantified on a scale from 0 - 4, as described previously (Rogers *et al.*, 2005; section

2.12). Both frozen and paraffin-embedded sections (*n* = 6 – 9 per group) were used to quantify corpus mucosal thickness and myofibroblast abundance whilst only paraffin-embedded sections (*n* = 3 – 5 per group) were used to quantify inflammation, epithelial defects, foveolar hyperplasia, oxyntic gland atrophy and antral Giemsa staining.

5.2.4 Statistics

For histopathological scores and Giemsa scores, data points represent individual animals and group data are presented as median ± range or IQR. Comparisons between experimental groups were made using a two-tailed Mann-Whitney *U*-test or Kruskal-Wallis ANOVA on ranks test with Dunn's post hoc analysis for multiple comparisons, and were considered significant at $P < 0.05$.

Antral and corpus mucosal thickness of individual animals was calculated as the mean of at least 10 measurements and group data are presented as mean ± SEM. Comparisons between experimental groups were made using a one-way ANOVA with Tukey's post hoc analysis for multiple comparisons, and were considered significant at $P < 0.05$.

5.3 Results

5.3.1 *H. felis* colonises antral mucosa but does not alter antral morphology of C57BL/6, PAI-1$^{-/-}$ and PAI-1-H/Kβ mice

Wild-type, PAI-1$^{-/-}$ and PAI-1-H/Kβ mice were chronically infected with *H. felis* to investigate the role of PAI-1 in *Helicobacter*-stimulated preneoplastic changes in the stomach. Bacterial colonisation was determined by quantifying Geimsa-stained *H. felis* in antral mucosa. The rationale behind investigating antrum, but not corpus, for colonisation was based on previous studies reporting that chronic *H. felis* infection results in antral-predominant colonisation in a number of mouse strains, including C57BL/6 (Sakagami *et al.*, 1996; Wang *et al.*, 1998; Takaishi *et al.*, 2009; Duckworth *et al.*, 2012). The antral glands of all C57BL/6 and PAI-1$^{-/-}$ mice were colonised by *H. felis* at 48 weeks post-inoculation. Colonisation of PAI-1$^{-/-}$ antrum tended to be elevated compared to C57BL/6 mice, but this did not reach statistical significance (Figure 5.1). Antral glands of all but one of the PAI-1-H/Kβ mice were colonised by *H. felis*. However, the mouse that was negative for *H. felis* colonisation did show phenotypic changes typical of infection suggesting that this particular mouse had been infected and had cleared the bacteria. There was no significant difference in antral *H. felis* colonisation of C57BL/6 and PAI-1-H/Kβ mice (Figure 5.2). Giemsa-stained antral tissues from age-matched uninfected mice were also examined and were found to be *H. felis* negative.

Antral mucosal morphology was similar in C57BL/6 and PAI-1$^{-/-}$ mice. Antral glands of both C57BL/6 and PAI-1$^{-/-}$ mice were slightly, but not significantly,

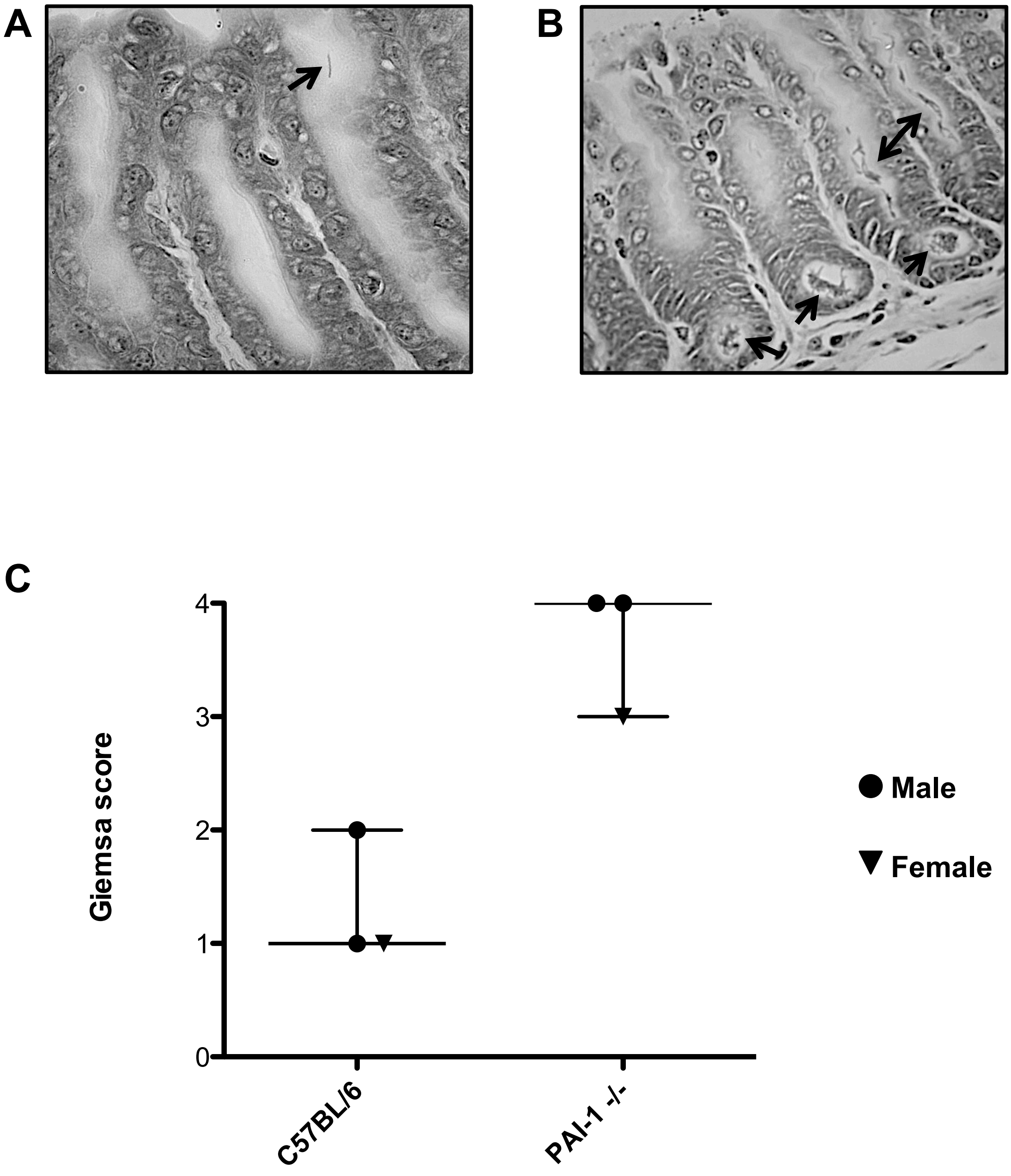


Figure 5.1. Colonisation of PAI-1$^{-/-}$ antral mucosa by *H. felis* tends to be elevated compared to wild-type 48 weeks after initial infection. A: Representative image of C57BL/6 antral mucosa colonised by Giemsa-stained *H. felis* 48 weeks after infection. Generally 1-2 bacteria were present in some antral gland lumen. **B:** Most PAI-1$^{-/-}$ antral gland lumen were colonised by many bacteria following 48 weeks of infection. *H. felis* colonies are illustrated by arrows, within the lumen of gastric glands. **C:** Antral mucosal colonisation with *H. felis* tended to be higher in PAI-1$^{-/-}$ compared to wild-type mice, but this did not reach statistical significance. All animals showed antral colonisation. Data for individual animals are shown in addition to group median ± range.

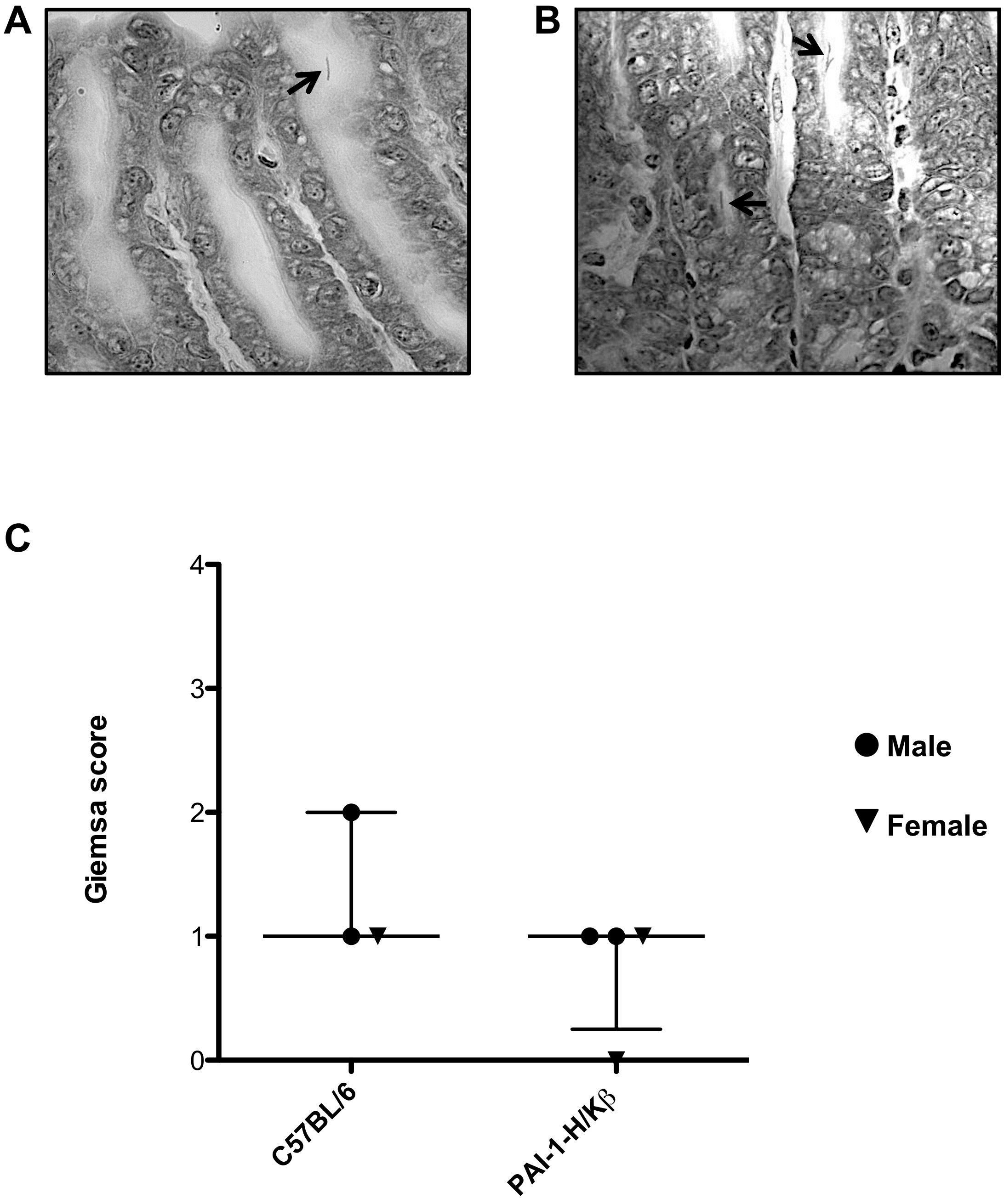


Figure 5.2. ***H. felis*** **colonises the antral mucosa of most PAI-1-H/Kβ mice 48 weeks after initial infection. A:** Colonisation of C57BL/6 antral mucosa by *H. felis* 48 weeks after infection. **B:** Similar to wild-type mice, some PAI-1-H/Kβ antral glands were colonised by 1-2 bacteria following 48 weeks of infection. **C:** Antral mucosa of all but one of the PAI-1-H/Kβ mice was colonised by *H. felis* 48 weeks after inoculation. The mouse lacking antral *H. felis* did show gastric phenotypic changes typical of infection however. There was no significant difference in antral *H. felis* colonisation of C57BL/6 and PAI-1-H/Kβ mice. Data for individual animals are shown in addition to group median ± IQR.

lengthened following *H. felis* infection, but morphology was otherwise unaltered (Figure 5.3). PAI-1-H/Kβ antral morphology was similar to C57BL/6 and was unchanged following *H. felis* infection (Figure 5.4).

5.3.2 Absence of PAI-1 protects against *H. felis*-stimulated corpus preneoplastic changes

Corpus mucosal morphology was similar in uninfected C57BL/6 and PAI-1$^{-/-}$ mice (Figure 5.5A & B). In response to chronic *H. felis* infection, C57BL/6 mice displayed severe histopathological changes typical of atrophic gastritis, including extensive inflammatory cell infiltration, mucosal thickening, foveolar hyperplasia, replacement of parietal and chief cell populations with mucous epithelium and glandular architectural abnormalities (Figure 5.5C & D). Corpus tissue from *H. felis* infected PAI-1$^{-/-}$ mice also showed marked inflammatory cell infiltration and had features typical of early progression to atrophic gastritis, including low-grade foveolar hyperplasia and occasional slight gland dilation. The chief cell population was depleted but the parietal cell population was largely preserved (Figure 5.5E & F).

Quantification of histopathological features revealed that PAI-1$^{-/-}$ mice were protected against the development of epithelial defects, foveolar hyperplasia, oxyntic gland atrophy, increases in corpus mucosal thickness and increases in corpus myofibroblast abundance in response to *H. felis* infection (Figure 5.6B - F).

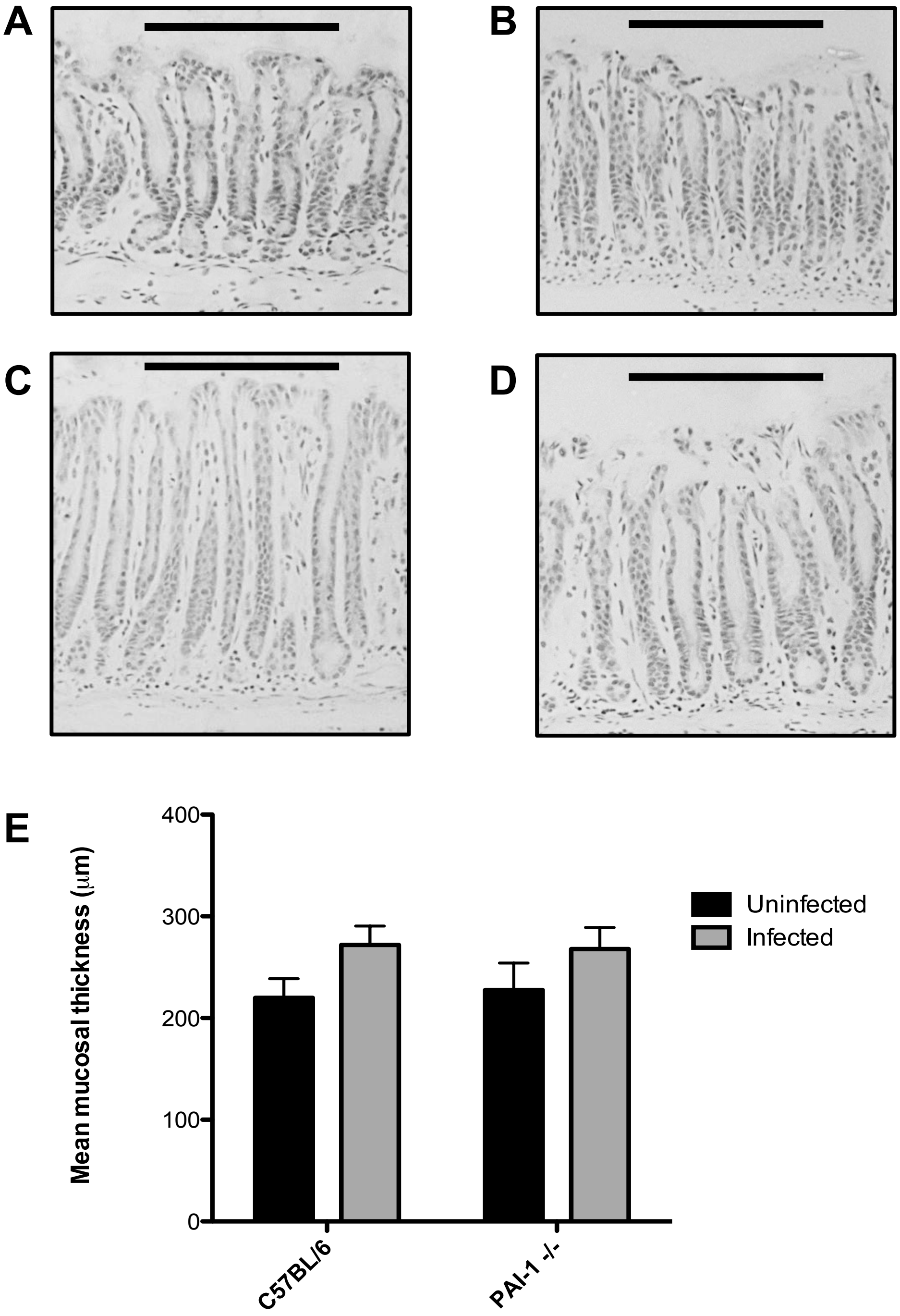


Figure 5.3. Antral morphology of wild-type and PAI-1$^{-/-}$ mice is similar, regardless of *H. felis* infection status. A: H&E stained section of uninfected C57BL/6 antrum. **B:** Uninfected PAI-1$^{-/-}$ antrum had similar morphology to wild-type antrum. **C:** *H. felis* infected C57BL/6 antrum had similar morphology to uninfected tissue, with slightly lengthened glands. **D:** *H. felis* infected PAI-1$^{-/-}$ antrum was also similar to uninfected tissue. **E:** Mean antral mucosal thickness of 53-56 week old C57BL/6 and PAI-1$^{-/-}$ mice was similar. Antral mucosal thickness of aged matched C57BL/6 and PAI-1$^{-/-}$ mice infected with *H. felis* for 48 weeks was slightly increased, but this did not reach significance. Data are expressed as mean ± SEM; $n = 3$ - 5 per group. Scale bars = 200μm.

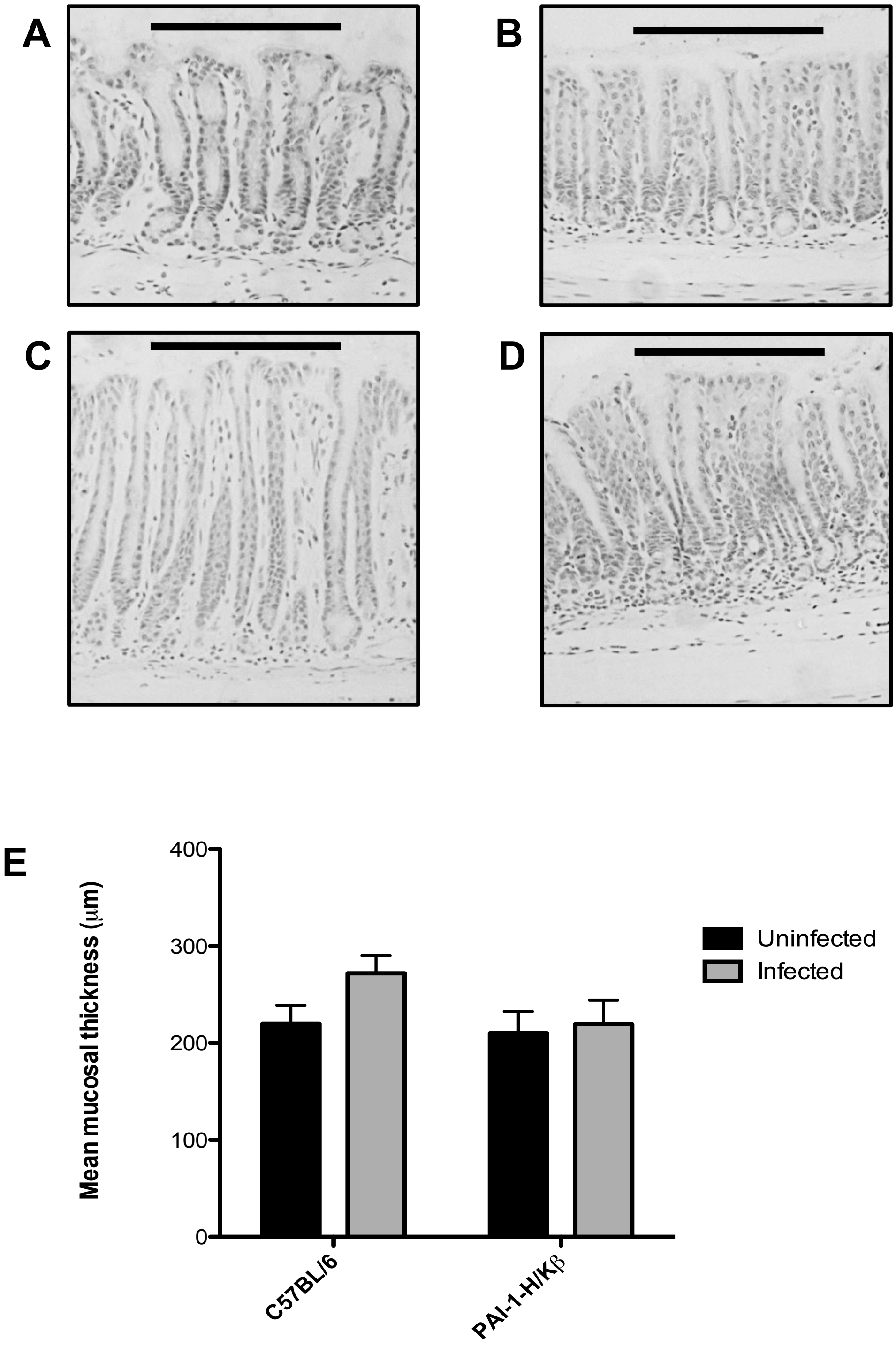


Figure 5.4. Wild-type and PAI-1-H/Kβ mice have similar antral morphology. **A:** Representative image of H&E stained uninfected C57BL/6 antrum. B: Uninfected PAI-1-H/Kβ antrum was similar to wild-type. **C:** *H. felis* infected C57BL/6 antrum, with similar morphology to uninfected tissue. **D:** Morphology of PAI-1-H/Kβ antrum was also unaffected by *H. felis* infection **E:** Mean antral mucosal thickness of 53-56 week old PAI-1-H/Kβ mice was equivalent to wild-type. Chronic *H. felis* infection had no effect on antral mucosal thickness. Data are expressed as mean ± SEM; *n* = 3 - 5 per group. Scale bars = 200μm.

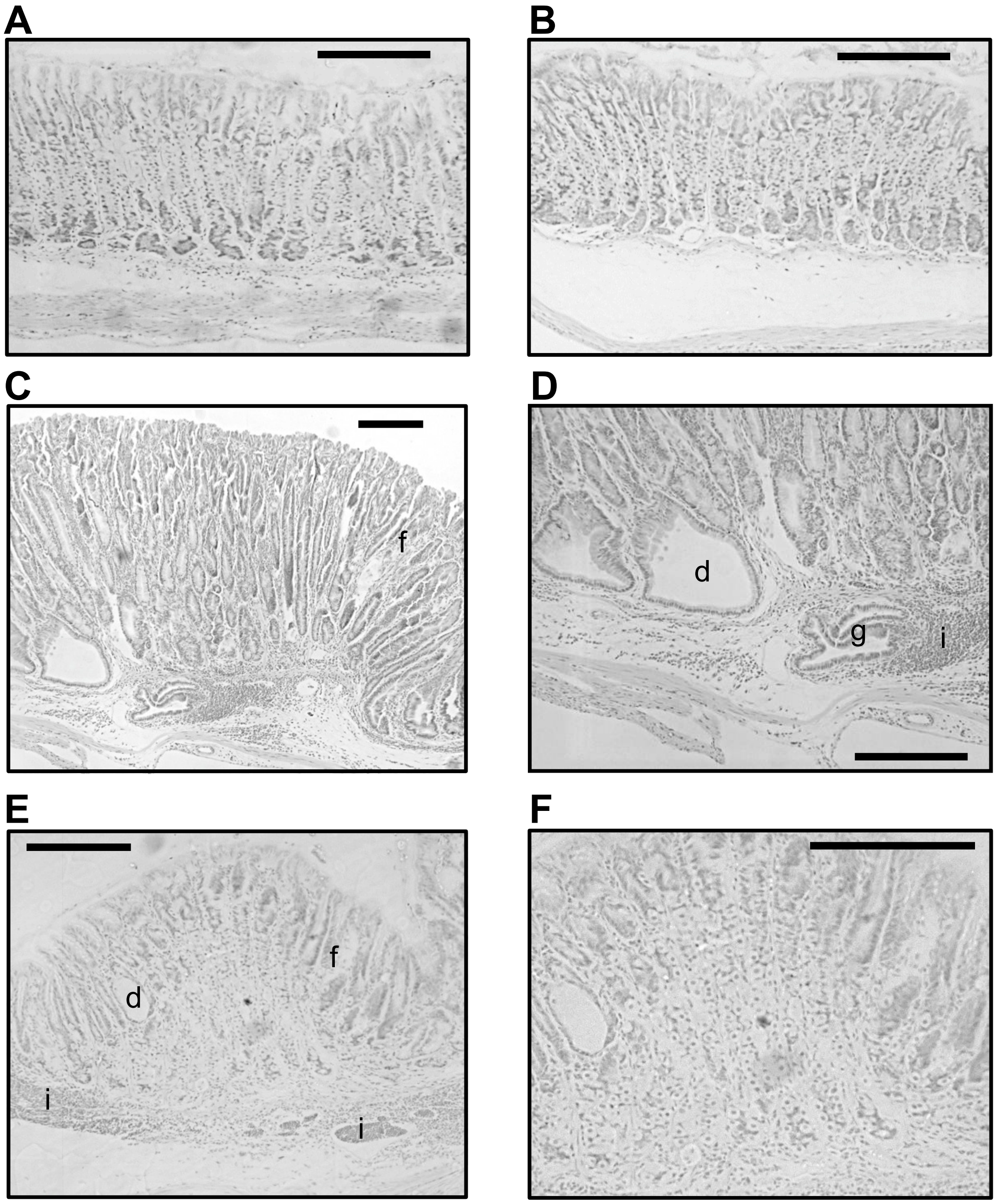


Figure 5.5. C57BL/6 mice show more severe corpus histopathological changes than PAI-1$^{-/-}$ mice in response to 48 weeks of *H. felis* infection. A: Representative H&E image of corpus tissue from an uninfected 53-56 week old C57BL/6 mouse. **B:** Age matched uninfected PAI-1$^{-/-}$ mice had similar corpus morphology to wild type mice. **C & D:** Corpus tissue from C57BL/6 mice infected with *H. felis* for 48 weeks was hypertrophic, with foveolar hyperplasia (**f**), replacement of parietal and chief cells with mucous epithelium, glandular dilation and distortion (**d**) and marked inflammatory cell infiltration, particularly as aggregates in the submucosa (**i**). Glandular regions had invaded into the submucosa (**g**). **E & F:** Corpus tissue from *H. felis* infected PAI-1$^{-/-}$ mice also showed marked inflammatory cell infiltration, particularly as submucosal aggregates (**i**), some lower grade foveolar hyperplasia (**f**) and occasional mild gland dilation (**d**). Parietal cells were largely preserved following infection in PAI-1$^{-/-}$ mice, whilst the chief cell population was diminished. Scale bars = 200μm.

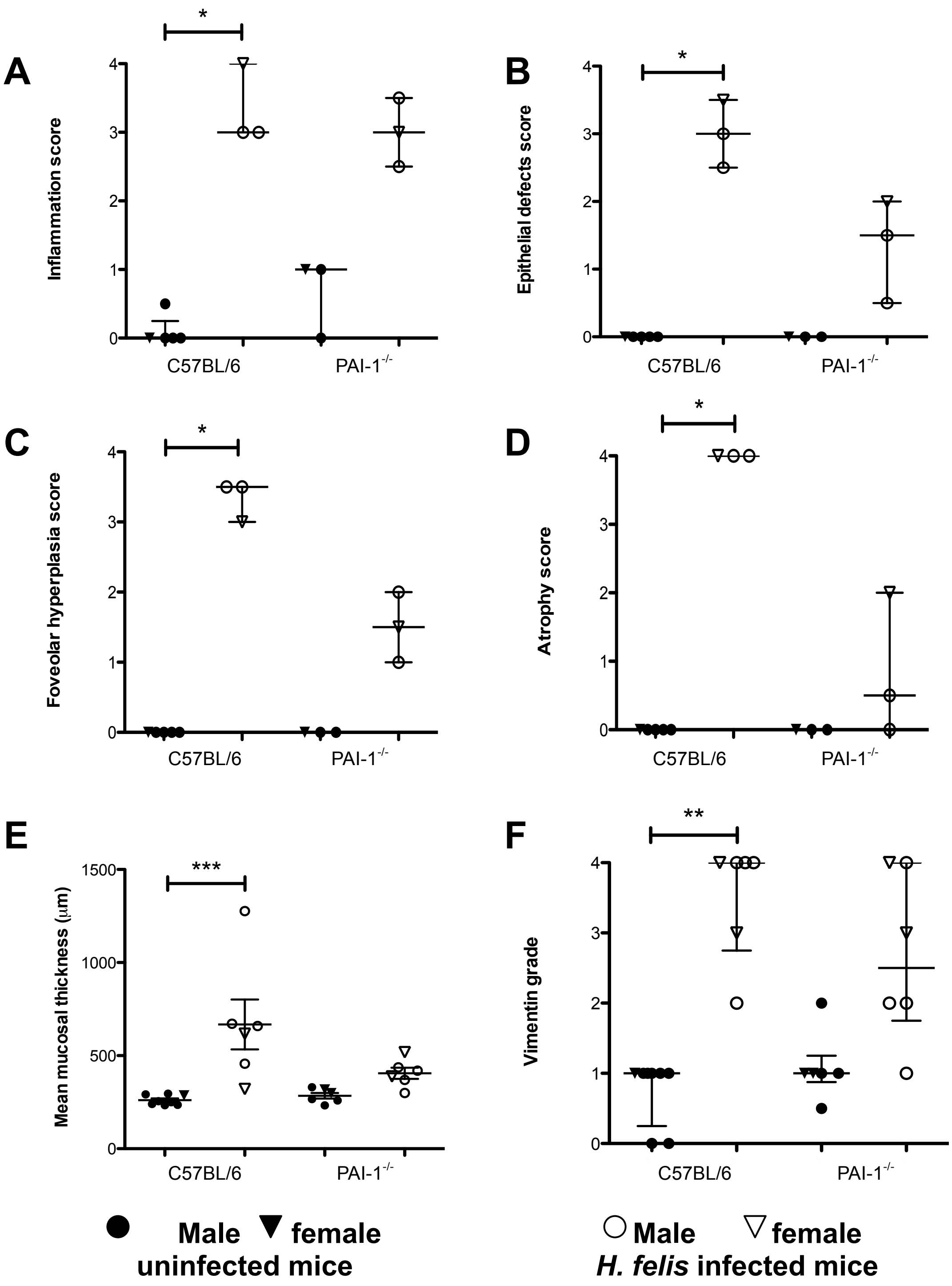


Figure 5.6. Absence of PAI-1 protects against *H. felis*-stimulated corpus preneoplastic changes. A: Chronic *H. felis* infection elicited inflammatory responses in wild-type and PAI-1$^{-/-}$ mice. Changes in inflammatory scores in infected PAI-1$^{-/-}$ mice compared to uninfected PAI-1$^{-/-}$ mice did not reach significance however. **B - F:** C57BL/6, but not PAI-1$^{-/-}$ mice, developed significant corpus epithelial defects, foveolar hyperplasia, glandular atrophy, increases in corpus mucosal thickness and increases in corpus mucosal myofibroblast abundance in response to 48 weeks of *H. felis* infection. Data for individual animals are shown in addition to median ± range or IQR, or mean ± SEM (mucosal thickness only). * $P < 0.05$, ** $P < 0.01$ (Kruskal-Wallis test); *** $P < 0.001$ (one-way ANOVA).

5.3.3 PAI-1-H/Kβ mice are protected against *H. felis*-stimulated corpus preneoplastic changes

In uninfected PAI-1-H/Kβ mice, the corpus mucosal thickness was increased compared with C57BL/6 mice and there was low-grade foveolar hyperplasia (Figure 5.7B). Corpus tissue of chronically infected PAI-1-H/Kβ mice had marked inflammatory cell infiltration and features indicative of early stages of progression towards atrophic gastritis, including low-grade foveolar hyperplasia and minor glandular structural defects. The chief cell population was depleted but parietal cells were preserved (Figure 5.7D).

Corpus histopathological scores revealed that distinct from wild-type mice, PAI-1-H/Kβ mice were protected against the development of *H. felis*-stimulated epithelial defects, foveolar hyperplasia, oxyntic gland atrophy, increases in mucosal thickness and increases in corpus mucosal myofibroblast abundance (Figure 5.8B - F).

5.4 Discussion

The work in this chapter builds upon previous reports of increased expression of PAI-1 by gastric epithelial cells in response to *H. pylori* infection (Herszenyi *et* al, 1997; Kenny *et al.*, 2008; Keates *et al.*, 2008; Ikeda *et al.*, 2009). PAI-1 is thought to modulate the tissue remodelling activity of the uPA system, so it was hypothesised that altering the expression of PAI-1 would modify the development of *H. felis* pathology. The data presented in this chapter provides novel insights into

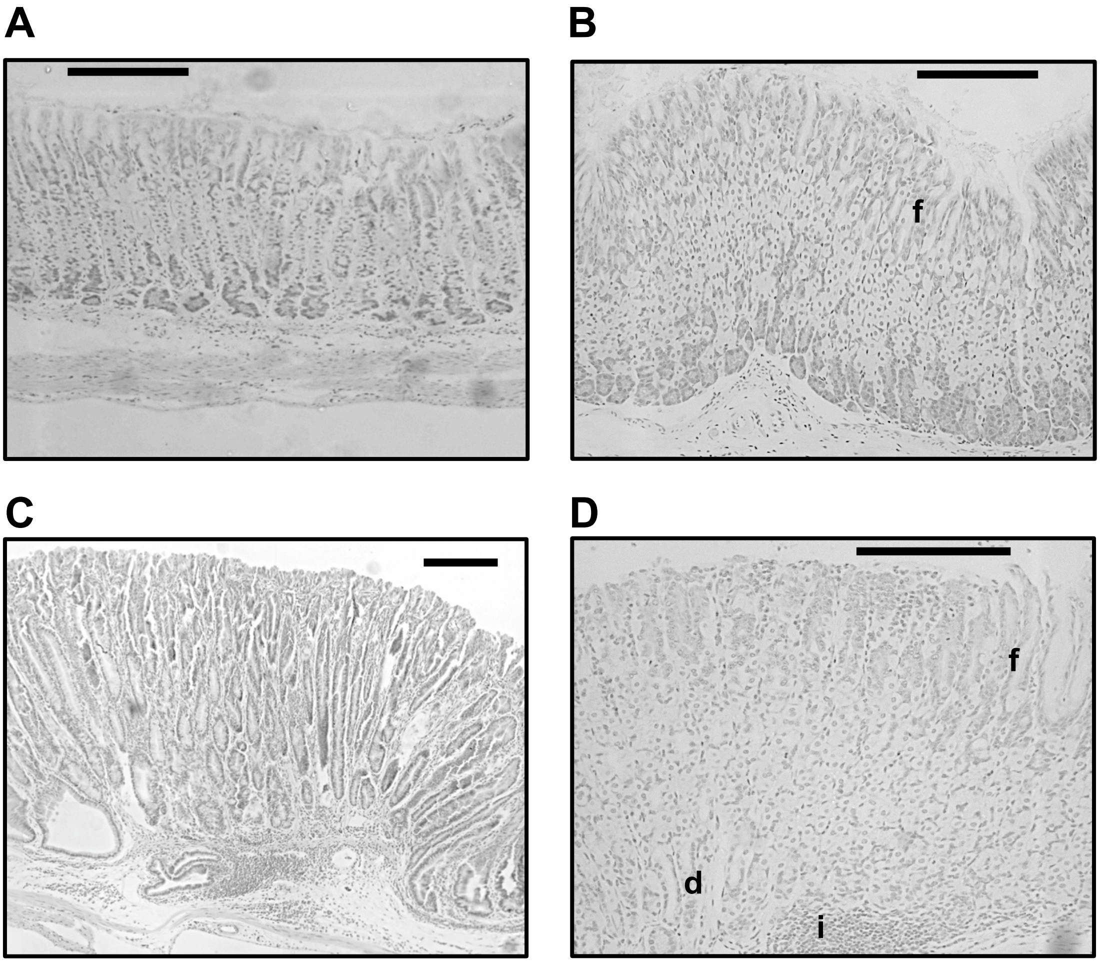


Figure 5.7. PAI-1-H/Kβ mice develop low-grade corpus histopathology in response to chronic *H. felis* infection. A: H&E stained corpus tissue from an uninfected 53-56 week old C57BL/6 mouse. **B:** Corpus tissue from age matched uninfected PAI-1-H/Kβ mice was hypertrophic compared to C57BL/6 corpus, with mild foveolar hyperplasia **(f)**. **C:** H&E stained C57BL/6 corpus tissue following 48 weeks of *H. felis* infection, showing features typical of atrophic gastritis. **D:** Corpus tissue from PAI-1-H/Kβ mice infected with *H. felis* for 48 weeks displayed marked inflammation (**i**), low grade-foveolar hyperplasia (**f**) and some mild glandular architectural defects (**d**). Parietal cells, but not chief cells, were preserved. Scale bars = 200μm.

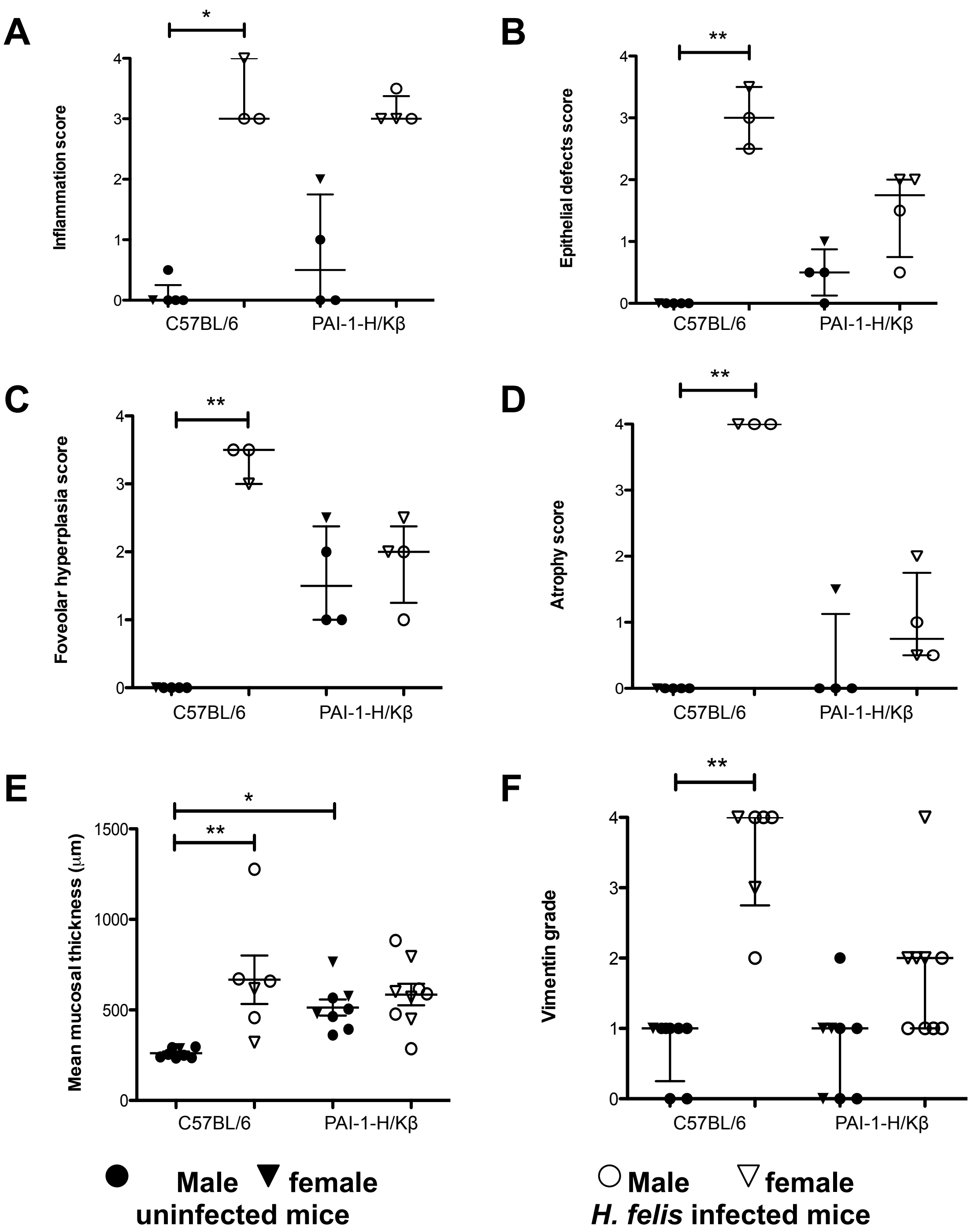


Figure 5.8. Increased gastric PAI-1 protects against *H. felis*-stimulated corpus preneoplastic changes. Chronic *H. felis* infection elicited inflammatory responses in wild-type and PAI-1-H/Kβ mice. Changes in inflammatory scores in infected PAI-1-H/Kβ mice compared to uninfected PAI-1-H/Kβ mice did not reach significance however. **B - F:** PAI-1-H/Kβ mice were protected against the development of corpus epithelial defects, foveolar hyperplasia, glandular atrophy, increases in corpus mucosal thickness and increases in corpus mucosal myofibroblast abundance in response to 48 weeks of *H. felis* infection. Data for individual animals are shown in addition to median ± range or IQR, or mean ± SEM (mucosal thickness only). * $P < 0.05$, ** $P < 0.01$ (Kruskal-Wallis test, or one-way ANOVA for mucosal thickness).

the role of PAI-1 in the development of *Helicobacter*-stimulated preneoplastic morphology in the stomach, with both PAI-1$^{-/-}$ and PAI-1-H/Kβ mice showing protection against corpus histopathological changes.

The results presented here are consistent with previous reports of the gastric morphological changes seen in C57BL/6 mice in response to chronic *H. felis* infection. The first study to investigate mucosal histological changes in response to 12 months of *H. felis* infection in C57BL/6 mice reported hyperplasia, associated with significant thickening of the mucosa resulting from lengthening of gastric pits and glands, marked parietal cell loss, glandular dilation, lymphocytic infiltration and glandular invasion into the submucosa (Fox *et al.*, 1996). These corpus phenotypic changes were replicated in C57BL/6 mice following 48 weeks of infection in this experiment. Similar results have been reported in studies examining corpus morphology following various durations of infection, including sustained active chronic gastritis and progressive histological changes characterised by mucosal hyperplasia, loss of parietal and chief cells, mucous cell metaplasia, gland dilation and, in some cases, cystic glandular invasion into submucosa (Sakagami *et al.*, 1996; Fox *et al.*, 1997; Wang *et al.*, 1998; Lichtenberger *et al.*, 1999; Dial *et al.*, 2000; Fox *et al.*, 2002; Court *et al.*, 2003; Cai *et al.*, 2005; Takaishi *et al.*, 2009; Schmitz *et al.*, 2011). Furthermore, the data on antral histology presented in this chapter are consistent with previously reported observations, describing preserved mucosal morphology in chronically *H. felis* infected C57BL/6 mice (Sakagami *et al.*, 1996). Some studies have reported a mild to moderate lengthening of antral glands, but no other histological changes in the antrum of C57BL/6 mice in response to *H. felis* infection (Fox *et al.*, 1997; Duckworth *et al.*, 2012). In this study, antral glands of *H. felis* infected C57BL/6 mice were slightly lengthened compared to uninfected animals, but this did not reach statistical significance.

Previous studies have highlighted the importance of gender in responses to *H. felis* infection of C57BL/6 mice. Female mice showed augmented inflammatory and histopathological responses to *H. felis* infection compared to male mice and, distinct from male mice, increased corpus epithelial cell proliferation and apoptosis in response to infection (Court *et al.*, 2003). Both male and female mice were used in this study and data from each were combined in comparative statistical analyses, as there were no major gender differences. Future experiments might include increased numbers of male and female mice, so that the effect of gender in PAI-1-modulated responses to *H. felis* infection might be revealed. Furthermore, given that the animals in this study were split into two groups for tissue processing, either for frozen sections or paraffin embedded sections, some of the analyses would benefit from increased animal numbers in each group. Nevertheless, where it was possible to assess the same parameter in both frozen and paraffin embedded sections, both groups yielded similar results, indicating that the analyses were robust and allowing data from the groups to be collated.

The data presented in this chapter suggest that expression of PAI-1 influences the degree of antral colonisation by *H. felis* 48 weeks after initial infection. Antral mucosae of PAI-1$^{-/-}$ mice tended to be colonised by greater numbers of *H. felis* than C57BL/6 mice. A recent study described a similar relationship between CD24 expression and *H. felis* colonisation, with CD24 null mice having a higher percentage of infected antral glands than wild-type mice (Duckworth *et al.*, 2012). This higher-degree of colonisation was associated with an attenuated corpus inflammatory response and protection against corpus hyperplasia and parietal cell atrophy. Previous studies have also described a positive relationship between retention of *H. felis* within the antral mucosa and attenuated corpus atrophic responses (Sakagami *et al.*, 1996; Wang *et al.*, 1998). It is likely that host-responses to *H. felis* infection that stimulate clearance of the bacteria also promote the development of glandular atrophy, and that these responses are at least partially mediated by PAI-1.

Both PAI-1$^{-/-}$ and PAI-1-H/Kβ mice were protected against the development of *H. felis*-stimulated increases in corpus mucosal thickness, oxyntic gland atrophy, foveolar hyperplasia and epithelial defects, implying that both absence of PAI-1 and increased PAI-1 expression protect against preneoplastic morphological changes in response to infection. This indicates that the balance between protective and aggressive mechanisms mediated by PAI-1 in *Helicobacter*-stimulated preneoplastic histopathogenesis is dependent on the levels of gastric PAI-1 expression, producing a U-shaped correlation between gastric PAI-1 expression and protection. These results are consistent with the “PAI-1 paradox” in cancer, described in section 1.6.6. This dose-dependent behaviour of PAI-1 can be explained by its complex biology, involving both uPA-dependent anti-proteolytic activity and mechanisms independent of uPA. At levels expressed in wild-type mice, deleterious mechanisms predominate, whilst at levels expressed by PAI-1-H/Kβ mice, protective mechanisms become dominant.

In terms of the potential mechanisms by which PAI-1 influences the gastric outcome of *H. felis* infection, an obvious candidate would be modulation of the immune response. Although both PAI-1$^{-/-}$ and PAI-1-H/Kβ mice demonstrated an inflammatory response to infection, further experiments are needed to determine the precise nature of cytokine and chemokine responses. It has been postulated that *H. felis* infection stimulates an autoimmune response against the corpus mucosa rather than a direct inflammatory response against the bacteria, hence the separation between the site of colonisation and the site of pathology (Sakagami *et al.*, 1996). An association between *H. pylori* infection and a gastric autoimmune response has been reported (Negrini *et al.*, 1991; Negrini *et al.*, 1997), mediated by a Th1 immune response (Bamford *et al.*, 1998). PAI-1 has been shown to modulate the balance between Th1 and Th2 immune responses in response to nasal allergy challenge, LPS and staphylococcal enterotoxin B, in favour of Th2 responses (Sejima *et al.*, 2005; Renckens *et al.*, 2006). uPA is required for the induction of both Th1 and Th2 immune responses (Gyetko *et al.*, 2002; Gyetko *et al.*, 2004). Therefore PAI-1 might act through uPA to modulate host immune responses.

A similar association between immune responses and expression of leptin has been reported. Similar to PAI-1, leptin is an adipokine that is also secreted by the gastric mucosa, expression of which is increased by *H. pylori* infection (Bado *et al.*, 1998; Breidert *et al.*, 1999; Azuma *et al.*, 2001). Leptin treatment has been shown to promote autoimmune encephalomyelitis in mice, switching the cytokine response from Th2 to Th1 profile (Matarese *et al.*, 2001). In terms of energy balance, PAI-1 and leptin have opposite effects; leptin is an anorexigenic agent, potentiating CCK-evoked satiety signals via vagal afferents, whilst PAI-1 endows resistance against these satiety signals (Matson *et al.*, 1997; Barrachina *et al.*, 1997; Wang *et al.*, 1997; Matson & Ritter, 1999; Emond *et al.*, 1999; Kenny *et al.*, 2013a). Similarly, at levels expressed by PAI-1-H/Kβ mice, PAI-1 might oppose the effects of leptin, promoting non-autoimmune Th2 responses, whilst at levels expressed by C57BL/6 mice, PAI-1 expression may favour the Th1 autoimmune profile. In common with other physiological and pathophysiological roles of PAI-1, regulation of immune responses is likely to be tissue-specific, as well as dose-dependent, which could account for the contrary observations seen in PAI-1 deficient mice in response to nasal allergy challenge, LPS and staphylococcal enterotoxin B compared to the hypothesised role in *Helicobacter* infection (Sejima *et al.*, 2005; Renckens *et al.*, 2006).

A number of studies point towards Th1 immune responses being an important modulator of *Helicobacter*-stimulated atrophic pathology and bacterial clearance. Production of IFN-γ by Th1 cells has been associated with *Helicobacter*-stimulated preneoplasia and clearance of the infection (Sayi *et al.*, 2009). Furthermore, suppression of Th1 responses by PGE2 has been shown to protect against *Helicobacter*-stimulated preneoplasia and promote persistent infection (Toller *et al.*, 2010). Induction of Th2 immune responses by concurrent helminth infection has been shown to be protective against *Helicobacter*-induced gastric atrophy, despite a robust inflammatory response and a high-degree of colonisation (Fox *et al.*, 2000; Du *et al.*, 2006), mirroring the response seen in PAI-1$^{-/-}$ mice. Characterisation of the cytokine and chemokine expression profiles of *H. felis* infected PAI-1$^{-/-}$, PAI-1-H/Kβ and C57BL/6 corpus tissue, by microarray and/or qPCR, would provide clarification as to whether PAI-1 modulates the immune response to infection. Furthermore, analysis of serum antibody responses to *H. felis* in C57BL/6, PAI-1$^{-/-}$ and PAI-1-H/Kβ mice would also be useful, to explore this hypothesis.

In terms of myofibroblast recruitment to the corpus mucosa in response to *H. felis* infection, both PAI-1$^{-/-}$ and PAI-H/Kβ mice had attenuated responses. This may be relevant to the reduced histopathological response seen in these mice. Previous studies have implied an important role for mucosal myofibroblasts in supporting epithelial and immune responses to *Helicobacter* infection, driving preneoplastic histopathogenesis. Studies investigating responses to *Helicobacter* infection in humans and in rodent models have identified myofibroblasts as a source of COX-2, bFGF and IGF-II, and as essential activators of Th17 cells, suggesting that myofibroblasts modulate inflammatory, immune and proliferative responses to infection (Fu *et al.*, 1999; Nakamura *et al.*, 2002; Kim *et al.*, 2004a; McCaig *et al.*, 2006; Pinchuk *et al.*, 2013). An examination of the microarray data generated in the previous chapter might reveal other gene products secreted by gastric myofibroblasts that could modulate epithelial and immune cell responses to *Helicobacter* infection. Future studies might include an analysis of global gene expression profiles of laser capture microdissected gastric myofibroblasts from chronically infected C57BL/6, PAI-1$^{-/-}$ and PAI-H/Kβ gastric mucosal tissues, to identify differentially expressed PAI-1-regulated transcripts and pathways that may be of significance to *Helicobacter*-stimulated pathology.

PAI-1 is profibrotic in various organs and is associated with increased myofibroblast abundance (Oda *et al.*, 2001; Matsuo *et al.*, 2005; Tuan *et al.*, 2008; Hu *et al.*, 2009; Senoo *et al.*, 2010; Bauman *et al.*, 2010; Ghosh & Vaughan, 2012). Conversely, and further illustrating the paradoxical nature of PAI-1 biology, aged PAI-1-deficient mice develop spontaneous cardiac fibrosis, associated with increased numbers of fibroblasts and endothelial-to-mesenchymal transition derived myofibroblasts (Ghosh *et al.*, 2010). The data presented in this chapter suggest that absence of PAI-1 and local increases in mucosal PAI-1 expression attenuate the recruitment of myofibroblasts in response to *Helicobacter* infection, providing novel insights into the relationship between PAI-1 and myofibroblast numbers in the stomach.

In summary, the data presented in this chapter indicate that gastric PAI-1 protects against *H. felis*-stimulated histopathogenesis at elevated levels expressed in PAI-H/Kβ mice, compared to wild-type mice, whilst absence of PAI-1 is also protective. This raises the question of whether PAI-1 protects the gastric mucosa against acute mucosal injury with a similar profile. This will be investigated in the next chapter.

5.5 Conclusions

1. Absence of PAI-1 facilitates persistent colonisation of the antral mucosa by *H. felis*.
2. Chronic *H. felis* infection does not alter antral mucosal morphology, regardless of PAI-1 expression.
3. Absence of PAI-1 protects against *H. felis*-stimulated preneoplastic histopathogenesis.
4. Elevated gastric expression of PAI-1 protects against *H. felis*-stimulated preneoplastic histopathogenesis.
5. Absence of PAI-1 and elevated gastric expression of PAI-1 protect against increases in corpus mucosal myofibroblast abundance in response to *H. felis* infection.

CHAPTER 6

ROLE OF THE uPA SYSTEM IN GASTRIC MUCOSAL RESPONSES TO INTRAGASTRIC INDOMETHACIN

6.1 Introduction

The uPA system modulates responses to tissue injury and regulates wound healing, through its roles in fibrinolysis and tissue remodelling, as described in section 1.6.4. The extracellular proteolytic activities of MMPs, and the inhibitory activities of tissue inhibitors of metalloproteinases (TIMPs), are known to regulate gastric mucosal injury, inflammation and healing. Expression of MMPs and TIMPs, and their activities, are altered in models of gastric mucosal injury, where MMP-2 and TIMP-1 are protective, whilst TIMP-2, MMP-1, MMP-3, MMP-9 and MMP-13 are deleterious (Baragi *et al.*, 1997; Lempinen *et al.*, 2000; Ganguly *et al.*, 2005; Kim *et al.*, 2011; Singh *et al.*, 2011; Pradeepkumar Singh *et al.*, 2011; Sharma *et al.*, 2012). However, little is known about the role of the uPA system in gastric mucosal protection, responses to gastric mucosal injury and gastric mucosal healing.

The myofibroblast is an important mediator of wound repair. Myofibroblasts respond to tissue injury by migrating into the damaged tissue, where they generate force to contract the wound, produce ECM components and secrete various paracrine factors, including extracellular proteases and their inhibitors, growth factors, cytokines, chemokines and lipid products, to support re-epithelialisation, epithelial and stromal cell function and ECM remodelling activity during the wound healing process (Mahida *et al.*, 1997; Serini & Gabbiani, 1999; Powell *et al.*, 1999; Wu *et al.*, 1999; Tomasek *et al.*, 2002). In the previous chapter, it was shown that both absence of PAI-1 and increased gastric expression of PAI-1 attenuated myofibroblast recruitment to the gastric mucosa in *H. felis* infection. The role of the uPA system in myofibroblast recruitment following gastric mucosal injury remains to be elucidated.

NSAIDs elicit gastric mucosal injury via attenuation of prostaglandin-mediated gastric cytoprotective mechanisms, direct cytotoxic effects and other prostaglandin-independent mechanisms (Wallace, 2008; Laine *et al.*, 2008, Takeuchi, 2012). Consequently, both topical indomethacin, via intragastric administration, and systemically administered indomethacin produce gastric mucosal injury (Djahanguiri, 1969; Morise *et al.*, 1999; Lempinen *et al.*, 2000).

Previous studies in this laboratory have demonstrated that PAI-1 is elevated in the gastric mucosa of aspirin and NSAID-treated patients and in ethanol and indomethacin-treated mice (Kenny *et al.*, 2013b). Furthermore, PAI-1 is protective in an ethanol-induced model of gastric mucosal injury, whereas elevated gastric uPA and absence of uPAR aggravate ethanol-induced lesion development (Kenny, 2008; Kenny *et al.*, 2013b). In this chapter, various mouse models are used to investigate the role of PAI-1, uPA and uPAR in the development of, and healing from, indomethacin-induced gastric mucosal injury.

6.1.1 Aims and objectives

The aim of the work in this chapter was to elucidate the role of the uPA system in responses to NSAID-induced injury and in healing of these lesions. The specific objectives were to:

1. quantify the development of indomethacin-induced gastric mucosal haemorrhagic lesions in PAI-1$^{-/-}$, PAI-1-H/Kβ, PAI-1$^{-/-,TG+}$, uPA-H/Kβ and uPAR$^{-/-}$ mice compared to wild-type mice;
2. determine the effects of exogenous PAI-1 on the development of indomethacin-induced gastric mucosal haemorrhagic lesions;

3. compare changes in gastric mucosal myofibroblast abundance during healing of indomethacin-induced gastric mucosal haemorrhagic lesions in C57BL/6, PAI-1$^{-/-}$, PAI-1-H/Kβ, PAI-1$^{-/-,TG+}$, uPA-H/Kβ and uPAR$^{-/-}$ mice.

6.2 Methods

6.2.1 Generation of PAI-1$^{-/-,TG+}$ mice

Mice expressing transgenic PAI-1 in the gastric mucosa, but otherwise null for PAI-1 (PAI-1$^{-/-, TG+}$) were generated by crossing PAI-1$^{-/-}$ with PAI-1-H/Kβ mice, as described in section 2.2.2.

6.2.2 Indomethacin-induced lesions

Male mice aged 10-13 weeks were dosed with indomethacin or vehicle, killed and stomachs prepared for lesion scoring, as described in section 2.2.4. In experiments involving PAI-1 or saline pre-treatment, animals were dosed by IP injection with 100µl of 2.5nmol/kg PAI-1 in saline (0.9% w/v NaCl) or saline alone, followed immediately by 20mg/kg intragastric indomethacin or vehicle.

6.2.3 Histology

Stomachs from indomethacin or vehicle treated mice were prepared for frozen sections followed by H&E staining, as described in section 2.3.

6.2.4 Immunohistochemical assessment of myofibroblast abundance

Male mice aged 10-12 weeks were dosed intragastrically with 10mg/kg indomethacin, 20mg/kg indomethacin or vehicle, and were killed 6 hours, 3 days, 5 days or 7 days after treatment. Stomachs were prepared for frozen sections (section 2.3) and sections prepared for immunohistochemical evaluation of vimentin abundance (sections 2.9 and 2.12). Similarly, some sections were prepared for desmin immunohistochemistry, applying the same grading system used for vimentin to quantify abundance.

6.2.5 Statistics

Results are presented both as individual data points and group mean ± SEM, or median ± range / interquartile range (IQR) for vimentin and desmin grades. Comparisons were made using an unpaired two-tailed Student's *t*-test or one-way ANOVA with Tukey's post hoc analysis for multiple comparisons, or Kruskal-Wallis ANOVA on ranks test with Dunn's post hoc analysis for multiple comparisons for vimentin and desmin grades, and were considered significant at $P < 0.05$.

6.3 Results

6.3.1 Intragastric indomethacin induces deep haemorrhagic lesions within the gastric mucosa

A model of indomethacin-induced gastric mucosal damage was employed to investigate the roles of PAI-1, uPA and uPAR using genetically modified mice. Initially, the protocol was optimised using C57BL/6 mice. In a previous study, intragastric indomethacin induced haemorrhagic lesions in a dose dependent manner from 5mg/kg to 20mg/kg and lesions were apparent between 6 and 18 hours after indomethacin administration (Morise *et al.*, 1999). In the present study, indomethacin administered by gavage at a dose of 20mg/kg produced significant lesions in C57BL/6 mice after 6 hours (Figure 6.2). There were multiple linear lesions, mostly within the corpus mucosa (Figure 6.1A). Histologically, these haemorrhagic lesions represented loci with deep mucosal injury and substantial epithelial cell death and exfoliation (Figure 6.1B).

6.3.2 Myofibroblast abundance during healing from indomethacin induced lesions

Myofibroblasts are known to be important mediators of wound healing, so their abundance in the gastric mucosa following indomethacin-induced injury was evaluated. The abundance of vimentin-expressing cells was not significantly different to untreated controls 3 or 5 days after intragastric indomethacin. After 7 days, there was a marked increase in vimentin-expressing cells within the corpus of 2 out of the 3 mice examined (Figure 6.3D). However, it became apparent during the course of these initial experiments that it would not be possible to continue with this procedure, owing to a mortality rate of >30% when animals were killed more than 24 hours after 20mg/kg

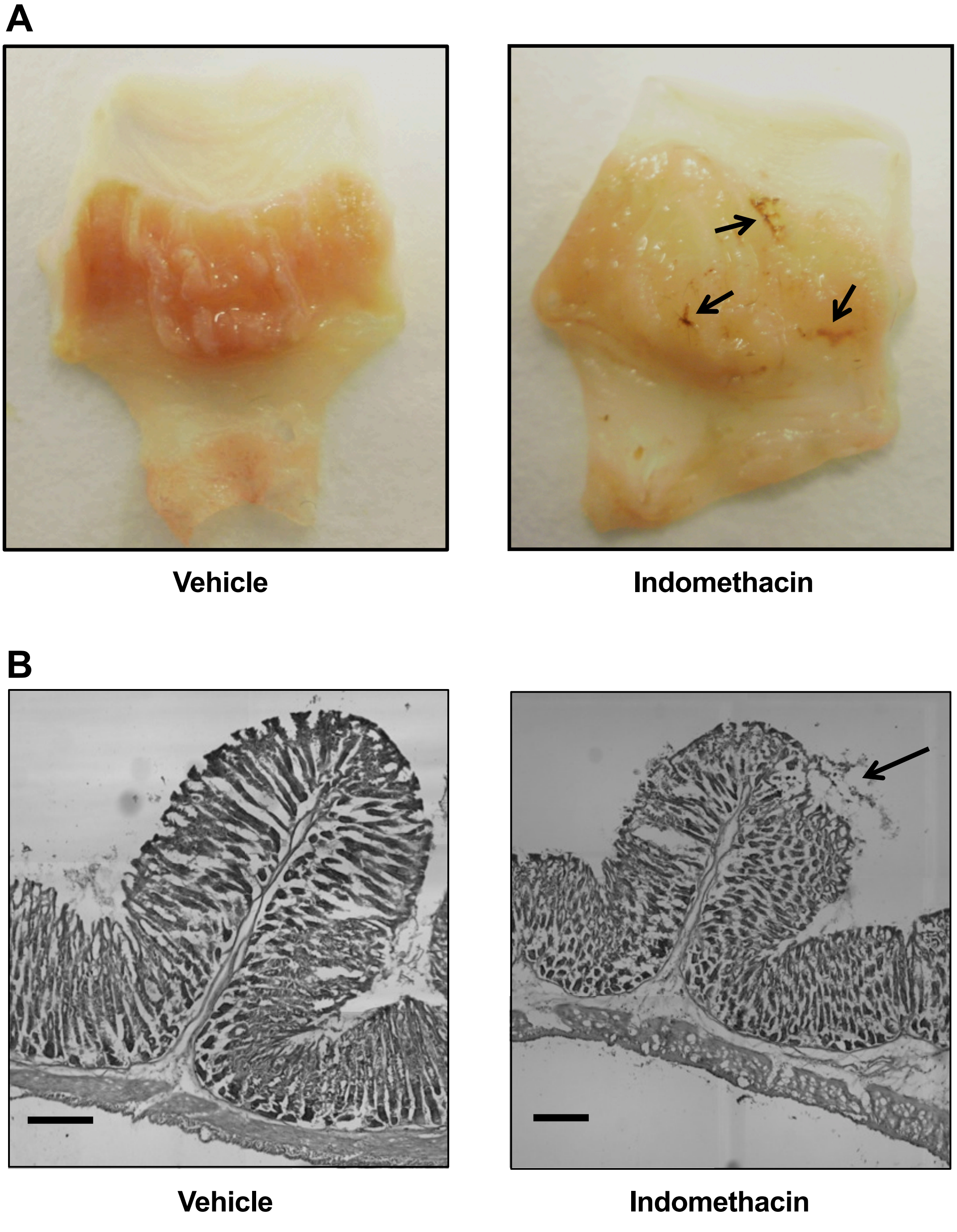


Figure 6.1. Deep mucosal haemorrhagic lesions develop following intragastric indomethacin. A: Multiple mucosal haemorrhagic lesions 6 hours after indomethacin treatment (arrows). The lesions were mostly limited to corpus tissue on all but the most severely injured tissues. **B:** H&E stained corpus from vehicle and indomethacin treated mice showed substantial epithelial cell loss and deep mucosal necrosis in haemorrhagic loci 6 hours after a 20mg/kg intragastric dose of indomethacin (indicated by arrow). Damage did not extend into the muscularis mucosae and submucosa. Scale bars = 200µm.

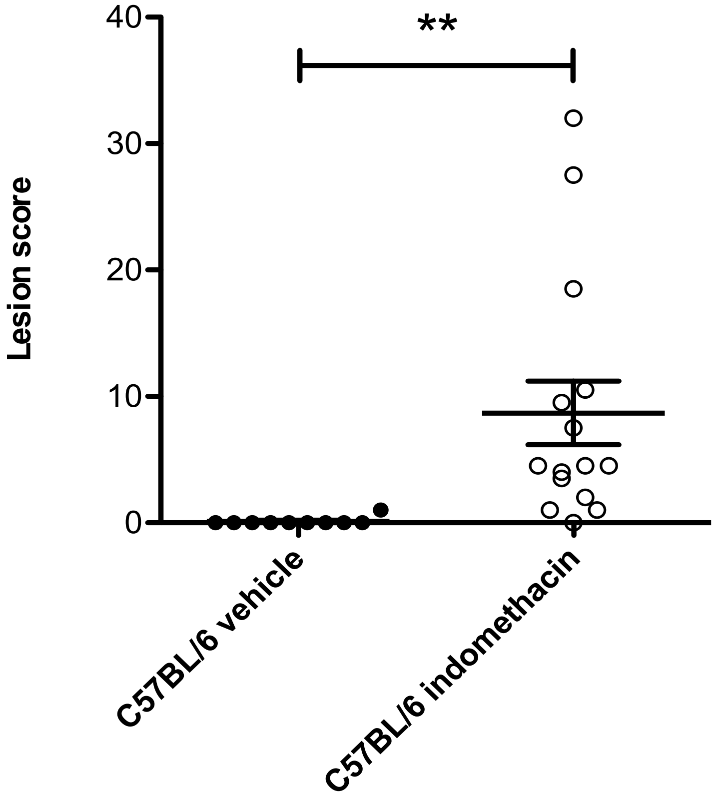


Figure 6.2 Intragastric indomethacin induces the development of significant haemorrhagic lesions. Lesion scores were significantly higher 6 hours after treatment with 20mg/kg indomethacin compared to vehicle treated controls. Data for individual animals are shown in addition to mean ± SEM. ** $P < 0.01$ (unpaired *t*-test).

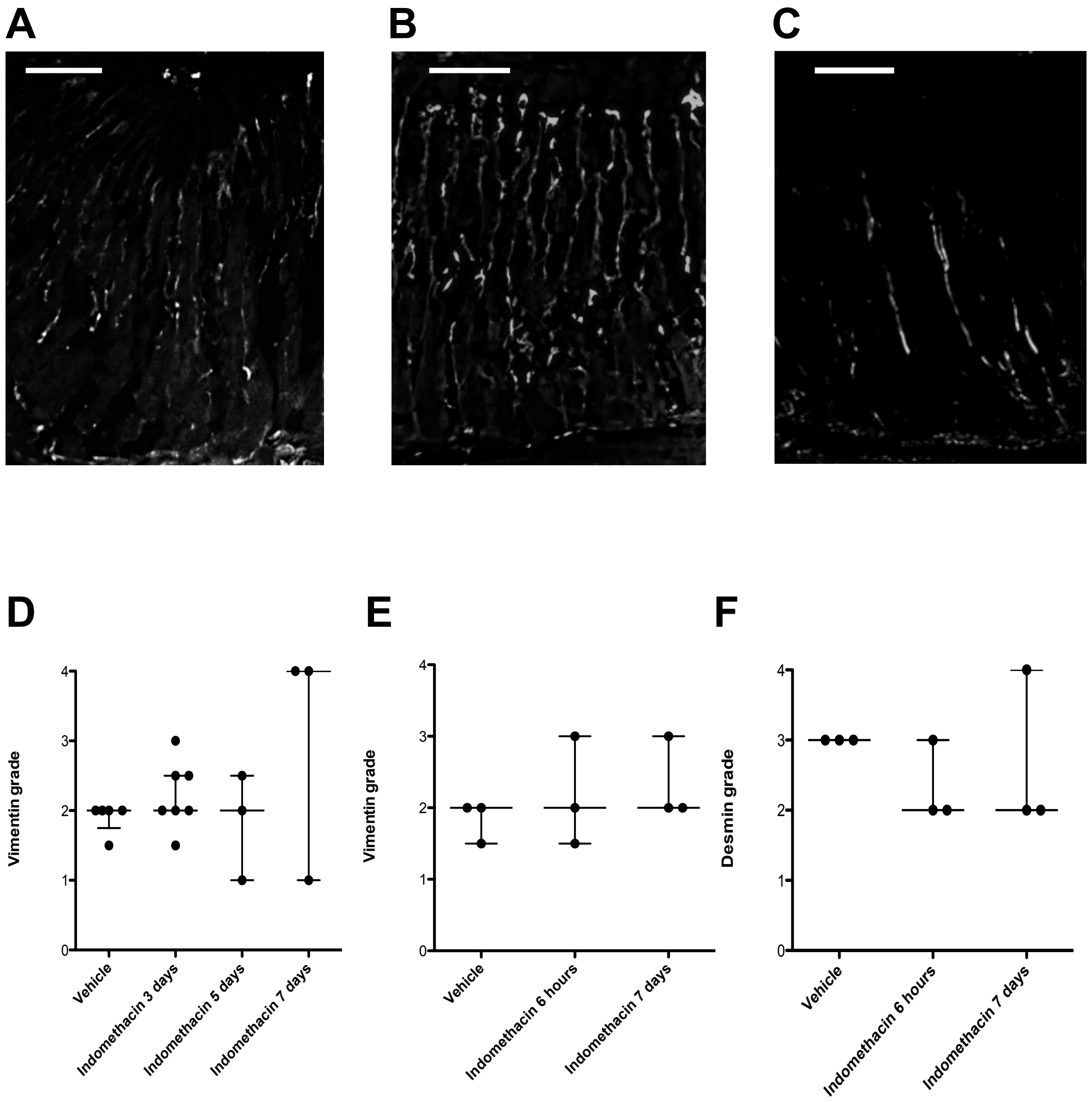


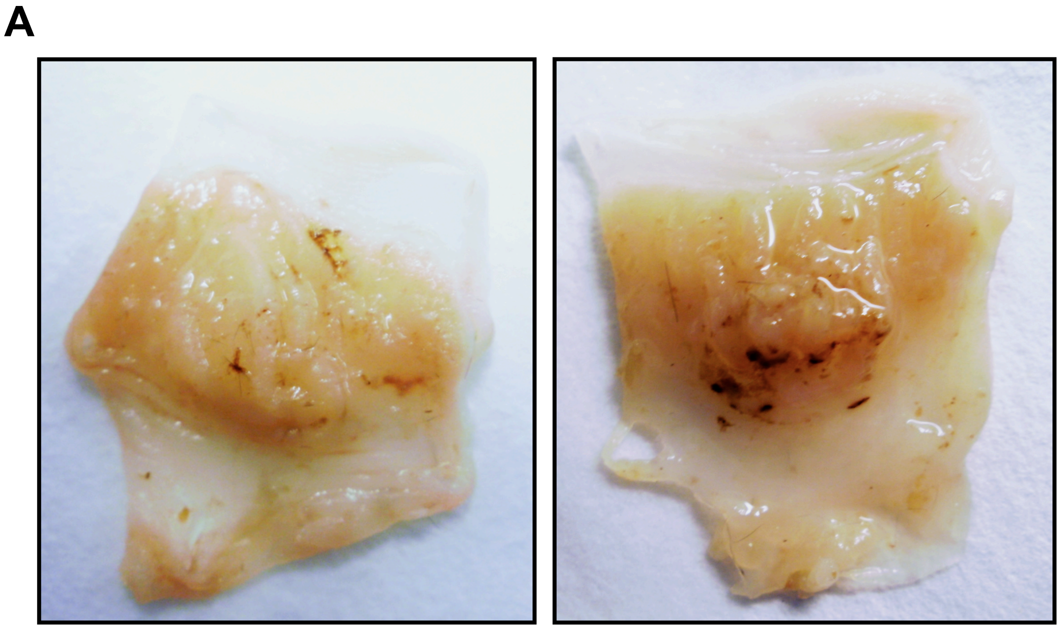
Fig 6.3. Increased corpus mucosal myofibroblast abundance 7 days after 20mg/kg intragastric indomethacin in some wild-type mice. A: Representative image of full corpus mucosal thickness vimentin immunostaining from a vehicle treated C57BL/6 mouse. **B:** Full corpus mucosal thickness vimentin distribution from a C57BL/6 mouse treated with 20mg/kg indomethacin, showing a marked increase in mucosal myofibroblasts 7 days after intragastric indomethacin. **C:** Representative image of full corpus mucosal thickness desmin staining, typically seen in all treatment groups. **D:** The abundance of corpus mucosal myofibroblasts markedly increased in 2 out of 3 C57BL/6 mice, 7 days after 20mg/kg indomethacin. **E:** In an experiment using 10mg/kg indomethacin to treat C57BL/6 mice, the abundance of corpus mucosal myofibroblasts remained unchanged up to 7 days. **F:** The abundance of corpus mucosal desmin expressing cells did not change significantly up to 7 days after intragastric indomethacin administration. Data for individual animals are shown in addition to median ± range or IQR. Scale bars = 100μm.

intragastric indomethacin. Post-mortem examination of the gastrointestinal tract revealed extensive intestinal bleeding in mice that had died during the course of these experiments. The experiment was repeated using 10mg/kg indomethacin, to investigate whether this dose of indomethacin could affect myofibroblast abundance during healing whilst eliminating mortality from the procedure. All animals survived following this dose of indomethacin. The abundance of both vimentin and desmin-expressing cells within the corpus mucosa was analysed by immunohistochemistry and was not significantly different to untreated controls 6 hours or 7 days after indomethacin (Figure 6.3E-F).

6.3.3 Gastric PAI-1 protects against indomethacin-induced mucosal injury

Three separate genetically modified mouse models were used to investigate the role of PAI-1 in the development of indomethacin-induced haemorrhagic lesions, using the protocol optimised in C57BL/6 mice. In PAI-1$^{-/-}$ mice, significant lesions were produced by intragastric indomethacin compared to vehicle treated controls and furthermore, these mice developed significantly more lesions in response to indomethacin than C57BL/6 mice (Figure 6.4).

To investigate the effect of increasing the expression of gastric PAI-1 on the development of indomethacin-induced gastric mucosal injury, the experiment was carried out in PAI-1-H/Kβ mice. PAI-1-H/Kβ mice did not develop significant lesions compared to vehicle treated controls (Figure 6.5), implying protection against injury by PAI-1.



C57BL/6 **PAI-1$^{-/-}$**

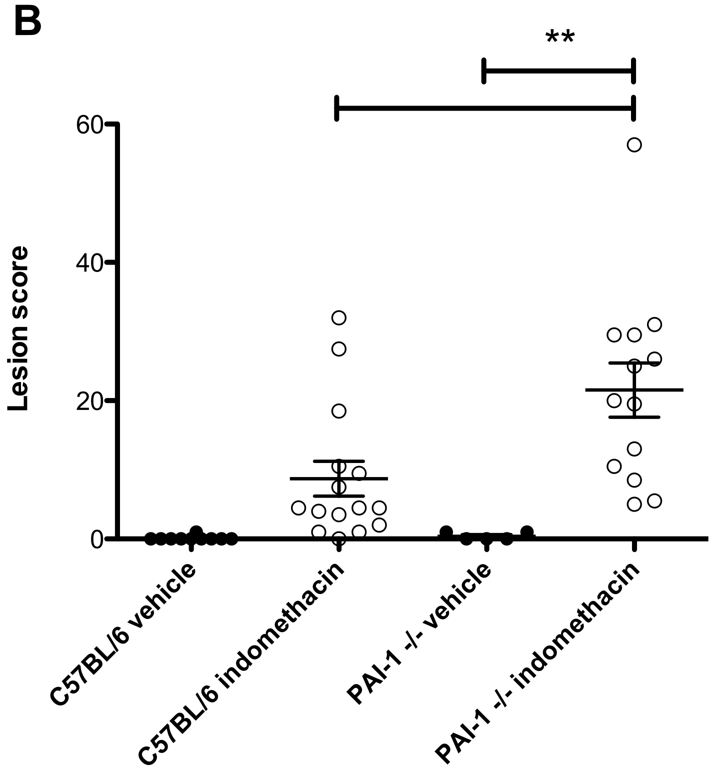


Figure 6.4. Absence of PAI-1 exacerbates the formation of indomethacin-induced haemorrhagic lesions. A: Representative images of stomachs 6 hours after intragastric administration of 20mg/kg indomethacin to wild type and PAI-1$^{-/-}$ mice. Generally PAI-1$^{-/-}$ mice had visibly more extensive mucosal injury than C57BL/6 mice. **B:** All PAI-1$^{-/-}$ mice developed lesions in response to intragastric indomethacin. PAI-1$^{-/-}$ mice, as a group, responded to indomethacin treatment with more severe gastric mucosal injury than wild-type animals. Data for individual animals are shown in addition to mean ± SEM. ** $P < 0.01$ (one-way ANOVA).

To further elucidate the role of PAI-1 encoded by the transgene in protection against indomethacin-induced gastric mucosal injury, PAI-1-H/Kβ and PAI-1$^{-/-}$ mice were crossbred, and the offspring backcrossed into PAI-1$^{-/-}$ mice to produce a strain in which only transgenic PAI-1 was expressed, i.e. null for wild-type PAI-1 (PAI-1$^{-/-, TG+}$). These mice developed significant lesions in response to intragastric indomethacin, with significantly higher lesions scores than C57BL/6 mice (Figure 6.6).

6.3.4 Exogenous PAI-1 exacerbates indomethacin-induced gastric mucosal injury

To further elucidate the action of PAI-1 in gastric mucosal protection, the effect of exogenous PAI-1 on the development of indomethacin-induced haemorrhagic lesions was investigated. C57BL/6 mice were dosed with either 2.5nmol/kg PAI-1 or saline by IP injection followed immediately by 20mg/kg indomethacin or vehicle by gavage. Mice treated with PAI-1 developed significantly more extensive mucosal damage than saline treated mice in response to intragastric indomethacin (Figure 6.7).

6.3.5 Increased gastric expression of uPA has no effect upon the development of indomethacin-induced gastric mucosal injury

To investigate the effect of increasing the expression of gastric uPA on the development of indomethacin-induced gastric mucosal injury, the experiment was carried out in uPA-H/Kβ mice. Utilising the same promoter used to drive expression of transgenic PAI-1 in PAI-1-H/Kβ mice, these mice express transgenic uPA in addition to wild-type uPA in gastric parietal cells. uPA-H/Kβ mice had a similar response to intragastric indomethacin as C57BL/6 mice in terms of lesion formation (Figure 6.8).

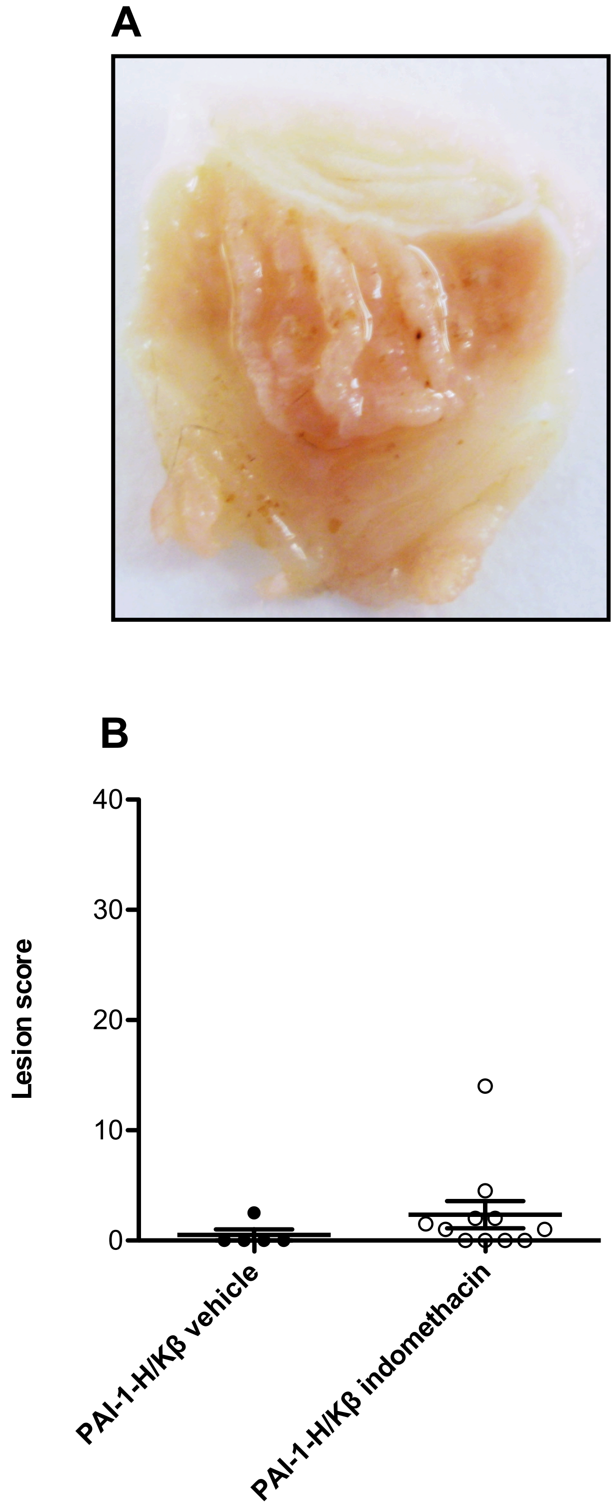


Figure 6.5 PAI-1-H/Kβ mice are protected against indomethacin-induced gastric mucosal injury. A: PAI-1-H/Kβ mice generally had visibly very weak mucosal injury or no visible injury in response to 20mg/kg intragastric indomethacin **B:** PAI-1-H/Kβ mice were protected against indomethacin-induced mucosal injury, with no significant increase in total lesion score compared to vehicle treated animals. Data for individual animals are shown in addition to mean ± SEM.

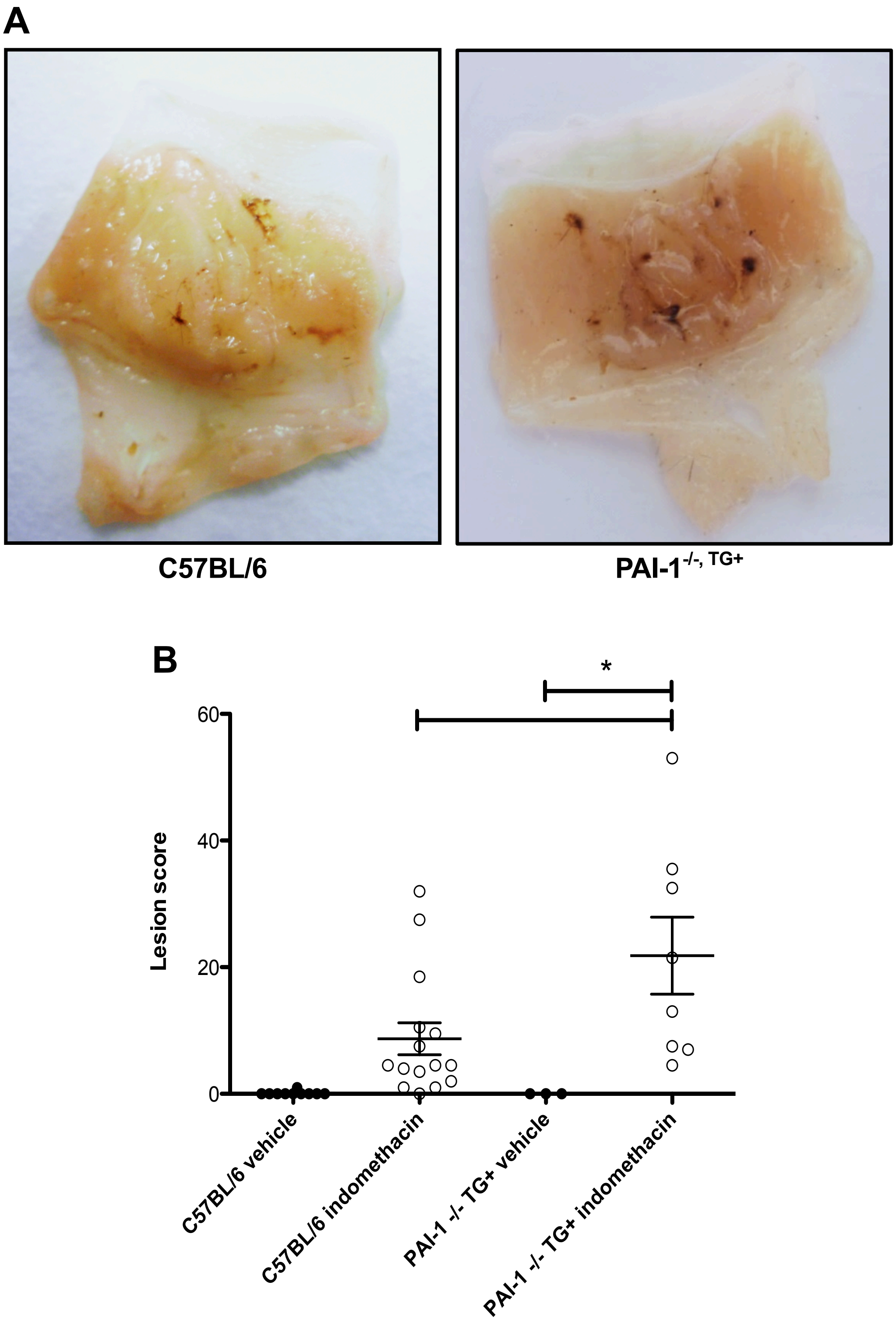


Figure 6.6. Transgenic PAI-1 alone does not convey protection against indomethacin-induced gastric mucosal injury. A: Representative images of stomachs 6 hours after an intragastric dose of 20mg/kg indomethacin to wild type mice and mice expressing gastric transgenic PAI-1 but not wild type PAI-1 (PAI-1$^{-/-,\ TG+}$). Generally PAI-1$^{-/-,\ TG+}$ mice had visibly more extensive mucosal injury than C57BL/6 mice. **B:** All PAI-1$^{-/-,TG+}$ mice developed haemorrhagic lesions in response to intragastric indomethacin. As a group, these mice developed more severe mucosal injury than wild type mice. Data for individual animals are shown in addition to mean ± SEM.* $P < 0.05$ (one-way ANOVA).

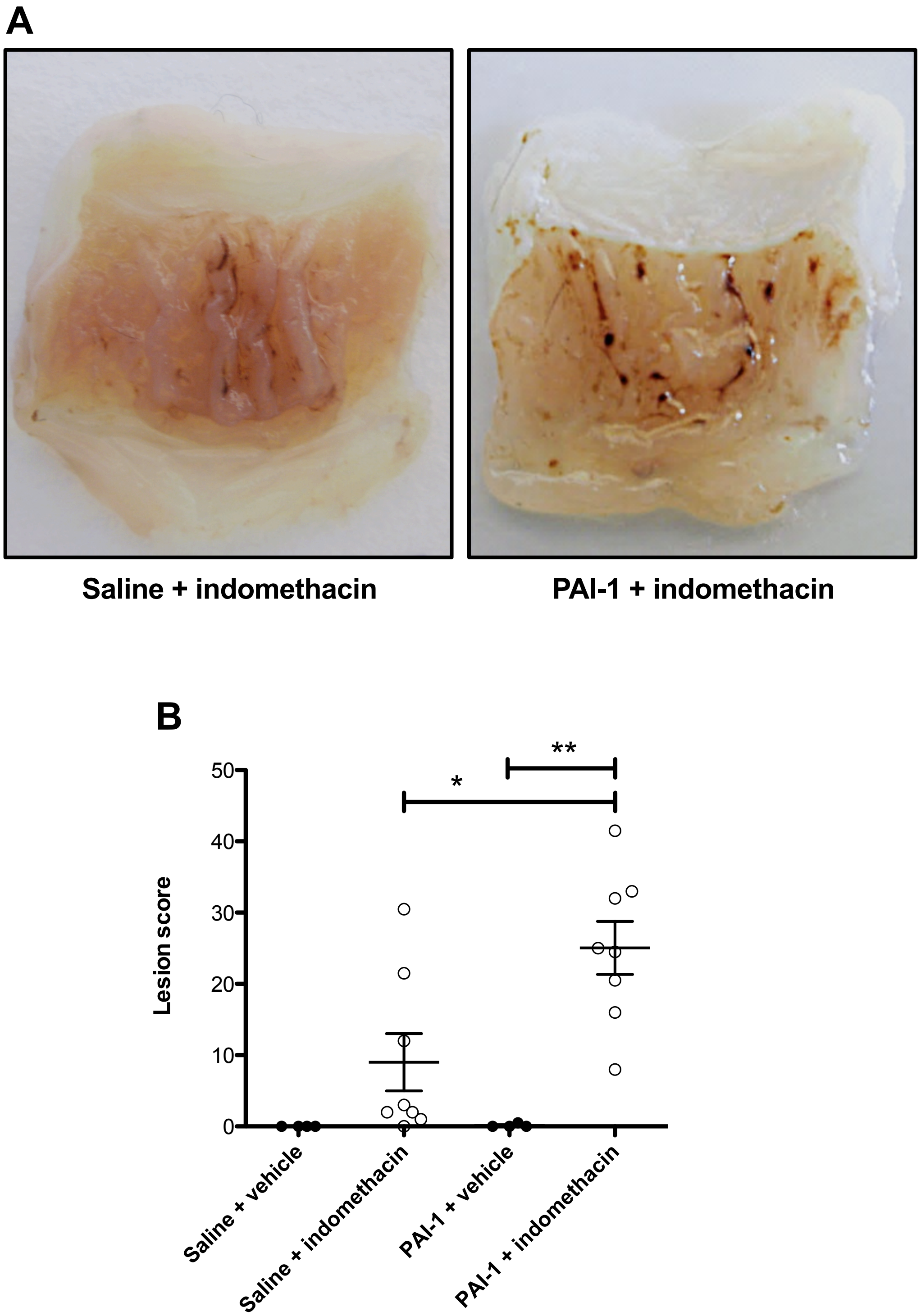


Figure 6.7 Exogenous PAI-1 exacerbates indomethacin-induced gastric mucosal injury. A: Representative images of C57BL/6 stomachs 6 hours after an IP injection of saline or 2.5nmol/kg PAI-1 immediately followed by 20mg/kg intragastric indomethacin. Generally mice treated with PAI-1 developed more extensive mucosal injury than mice treated with saline. **B:** All mice treated with PAI-1 developed haemorrhagic lesions in response to intragastric indomethacin. As a group, mice treated with PAI-1 developed significantly more indomethacin-induced lesions than those treated with saline. Data for individual animals are shown in addition to mean ± SEM. * $P < 0.05$, ** $P < 0.01$ (one-way ANOVA).

6.3.6 Absence of the uPA receptor tends to exacerbate indomethacin-induced gastric mucosal injury.

To elucidate the role of uPAR in the development of indomethacin-induced lesions, uPAR$^{-/-}$ mice were dosed with indomethacin. Significant lesions were produced by intragastric indomethacin in uPAR$^{-/-}$ mice, compared to vehicle treated mice (Figure 6.9B). There was much variability in the lesion scores of uPAR$^{-/-}$ mice in response to indomethacin, with some animals developing a high degree of mucosal damage, and generally uPAR$^{-/-}$ mice developed more extensive lesions than C57BL/6 mice (Figure 6.9A & B). This did not reach statistical significance however (Figure 6.9B).

6.4 Discussion

NSAID-induced gastrointestinal bleeding is a significant clinical issue. Hospitalisations due to haemorrhagic ulcer complications are rising amongst the elderly population, in which NSAID use is most prevalent (Higham *et al.*, 2002). The annual incidence of upper gastrointestinal complications associated with NSAID use is approximately 1%-1.5%, and mortality rates due to chronic NSAID treatment are estimated at between 0.045% and 0.083% (Tramer *et al.*, 2000; Lanas *et al.*, 2005; Laine *et al.*, 2008). The uPA system critically regulates responses to tissue injury via its effects on fibrinolysis and tissue remodelling, yet little is currently known about the role of uPA, uPAR and PAI-1 in gastric mucosal injury and gastric mucosal healing. In a previous study, gastric PAI-1 was shown to be protective against gastric mucosal damage induced by ethanol, whereas increased gastric uPA expression and absence of uPAR both exacerbated lesion development

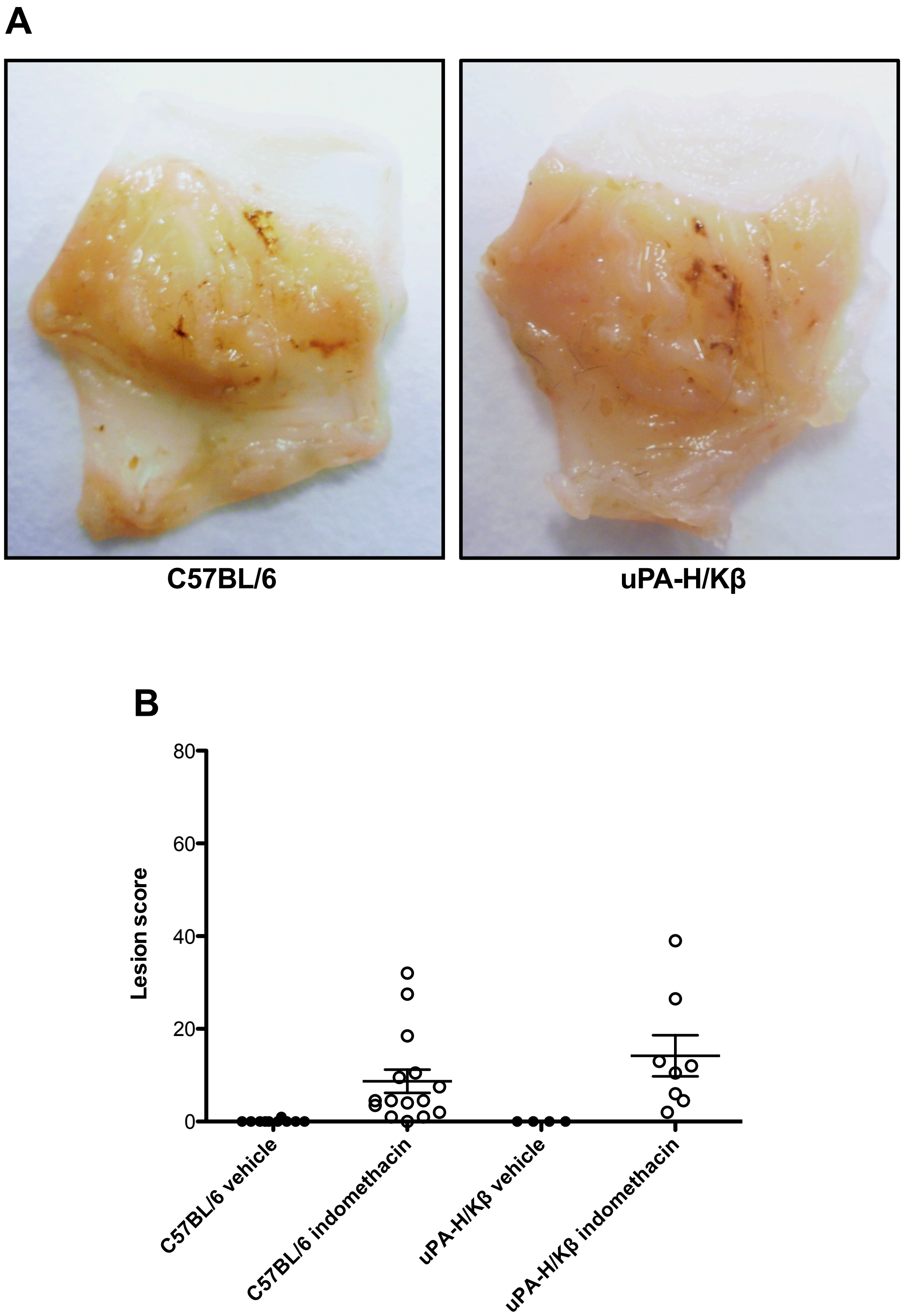
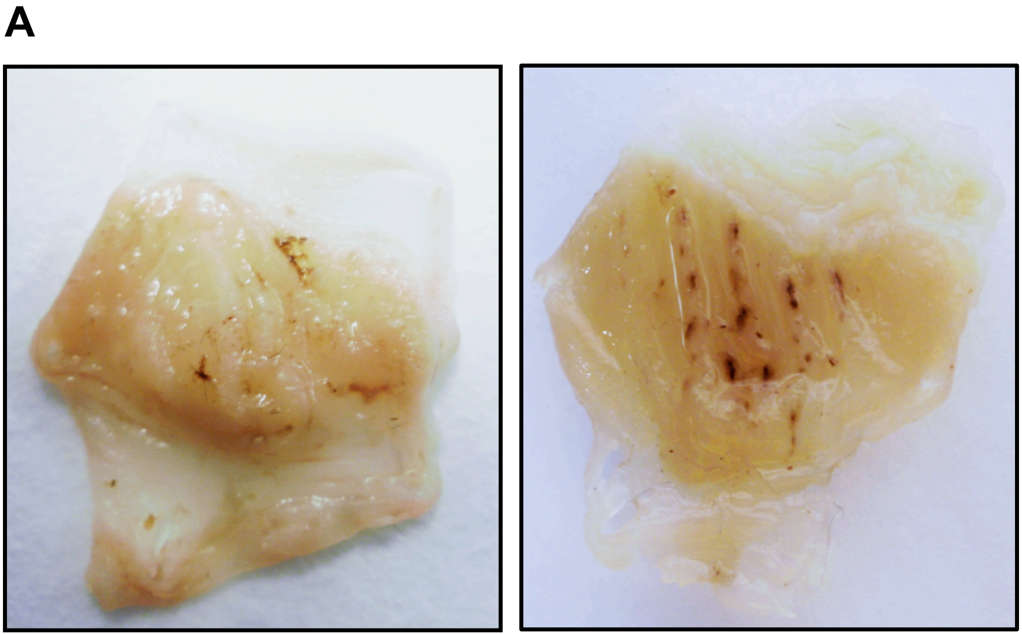


Figure 6.8 uPA-H/Kβ mice have similar responses to intragastric indomethacin as wild-type mice. A: Representative images of stomachs 6 hours after an intragastric dose of 20mg/kg indomethacin to C57BL/6 and uPA-H/Kβ mice, showing a similar extent of mucosal damage in both strains. **B:** Wild-type and uPA-H/Kβ mice develop lesions to a similar degree. Data for individual animals are shown in addition to mean ± SEM.



C57BL/6 uPAR$^{-/-}$

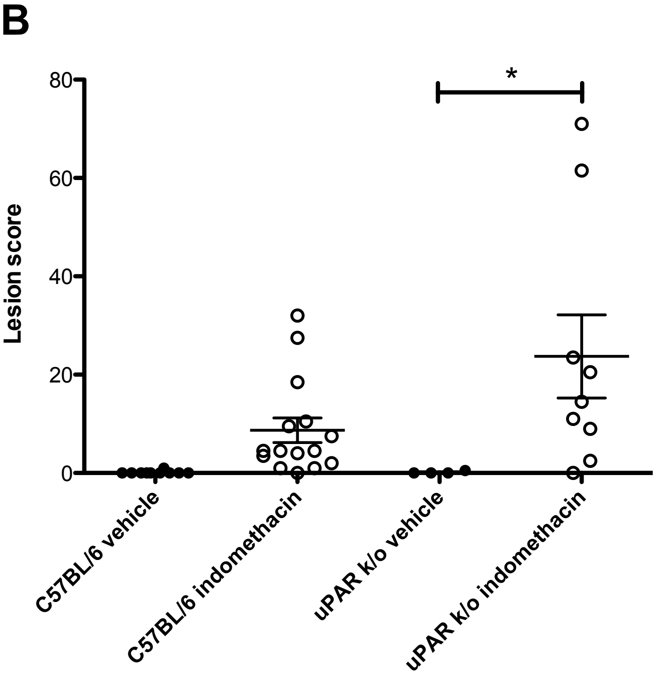


Figure 6.9 uPAR$^{-/-}$ mice tend to develop more extensive gastric mucosal damage in response to intragastric indomethacin. A: Representative images of C57BL/6 and uPAR$^{-/-}$ stomachs 6 hours after 20mg/kg intragastric indomethacin. Visible mucosal injury of uPAR$^{-/-}$ stomachs was generally more extensive than wild type. **B:** Some uPAR$^{-/-}$ mice developed severe mucosal injury in response to intragastric indomethacin. However, the elevation in mean total lesion score of uPAR$^{-/-}$ mice as a group compared to wild type did not reach significance, owing to a large degree of variability in this strain. Data for individual animals are shown in addition to mean ± SEM. * $P < 0.05$ (one-way ANOVA).

(Kenny, 2008; Kenny *et al*., 2013b). The work presented in this chapter points to a role for PAI-1 in protection against indomethacin-induced gastric mucosal injury.

Mucosal expression of uPA and PAI-1 has been shown to be increased and tPA expression decreased at the site of gastric and duodenal ulcers, with uPA being highest in patients with the greatest bleeding tendency (Wodziński *et al.*, 1993; Herszenyi *et al.*, 1997). Earlier studies provided evidence of increased mucosal plasminogen activator activity at gastric ulcer sites and increased plasma PAI-1 in patients with gastric ulcer (Cox *et al.*, 1967; Kurose *et al.*, 1990). Together these results imply a role for the fibrinolytic system in gastric mucosal injury. Furthermore, increased fibrinolytic activity of gastric juice and of the mucosa has been reported in gastroduodenal ulcer haemorrhage and elevated fibrinolysis has been associated with adverse outcomes in patients with upper gastrointestinal bleeding (Nilsson *et al.*, 1975; Vreeburg *et al.*, 2001; Gutierrez *et al.*, 2001). Inhibition of fibrinolysis, which was significantly elevated at the ulcer edge mucosa by plasminogen activator activity, attenuated haemorrhage in a rat model of gastric ulcer (Stenberg *et al.*, 1983a; Stenberg *et al.*, 1983b). The findings presented in this chapter, together with evidence from previous studies, suggest that PAI-1 restrains fibrinolysis thereby decreasing haemorrhagic gastric mucosal injury.

The importance of PAI-1 in stabilising the haemostatic response *in vivo* is highlighted by the existence of bleeding disorders in patients with PAI-1 deficiency (Schleef *et al.*, 1989; Diéval *et al.*, 1991; Lee *et al.*, 1993; Minowa *et al.*, 1999; Kuhli *et al.*, 2005). PAI-1 is produced by platelet aggregates in the thrombus and binds platelet-associated fibrin, rendering it resistant to lysis and so maintaining the integrity of the clot (Braaten *et al.*, 1993). Increased bioavailability of PAI-1 at haemorrhagic loci might optimise the fibrinolytic response for haemostasis and rapid remodelling of the wound towards healing. Absence of PAI-1 may facilitate persistent fibrinolysis and maintain haemorrhage at lesion loci, accounting for the widespread lesions seen in PAI-1$^{-/-}$ gastric mucosa 6 hours after indomethacin treatment.

One possibility for the similarities between gastric mucosal responses to indomethacin in wild-type and uPA-H/Kβ mice is that a compensatory reduction in gastric tPA expression, either as a result of expression of the uPA transgene or following indomethacin treatment, results in no net difference in the rate of fibrinolysis in the gastric mucosa of uPA-H/Kβ mice. To address this possibility, D-dimer ELISA was performed on plasma samples from each of the treatment groups, to assess fibrinolytic activity following indomethacin treatment of each mouse strain. Unfortunately, this assay failed to yield meaningful results. An evaluation of gastric tPA expression by uPA-H/Kβ mice may help to clarify this point. Alternatively, gastric mucosal fibrinolytic activity in response to indomethacin treatment of each mouse strain could be determined by incubating samples of the gastric mucosa on fibrin plates (Jespersen & Astrup, 1983).

An alternative hypothesis for the mechanism through which the uPA system exerts its effects on gastric mucosal injury is by modulating vascular permeability. Previous studies demonstrated that plasmin inhibitors attenuated the development of gastric mucosal lesions in response to 50% ethanol and 1% ammonia in rats and that this protection was associated with suppression of the increase in mucosal vascular permeability, independent of a prostaglandin-mediated mechanism (Murakami *et al.*, 1989; Yoo *et al.*, 1989). Furthermore, antithrombin-III has been shown to attenuate fibrinolytic activity and increased microvascular permeability and also protect against gastric mucosal injury in response to repeated electrical stimulation of the small arterial wall (Kurose *et al.*, 1994). This suggests a role for plasmin in the development of gastric mucosal injury. The inhibitory activity of PAI-1 in the generation of plasmin may therefore account, at least in part, for the protective role of PAI-1, via a reduction in plasmin-induced increases in mucosal vascular permeability in response to indomethacin.

In order to elucidate the role of gastric PAI-1 in cytoprotection, indomethacin-induced lesion formation in response to transgenic PAI-1 alone was investigated. Responses to intragastric indomethacin in PAI-1$^{-/-, \text{TG+}}$ mice were similar to PAI-1$^{-/-}$ mice, implying that the contribution of wild-type PAI-1 to the overall expression of PAI-1 in the stomachs of PAI-1-H/Kβ mice is essential for the gastric cytoprotective phenotype seen in this strain. Furthermore, expression of the transgene alone does not produce enough PAI-1 in the stomach to endow any protection against indomethacin-induced lesion development, rendering these mice more susceptible to gastric mucosal injury than wild-type mice. In a previous study, it was shown that gastric tissue of PAI-1-H/Kβ mice expresses significantly more total PAI-1 mRNA than that of wild-type mice (Kenny *et al.*, 2013a). It would be useful to extend this analysis to look at total PAI-1 mRNA expression by gastric tissues of PAI-1$^{-/-}$ and PAI-1$^{-/-, \text{TG+}}$ mice and also to quantify the expression of total PAI-1 protein by gastric tissues of wild-type, PAI-1$^{-/-}$, PAI-1-H/Kβ and PAI-1$^{-/-, \text{TG+}}$ mice, to establish whether responses to indomethacin are directly linked to the generation of PAI-1 by gastric tissues.

Contrary to the effects of increased gastric PAI-1, exogenous PAI-1 exacerbated lesion development when administered by IP injection. There are a number of issues that need to be kept in mind in interpreting these observations. For one thing, the present studies used a stabilised mutant of human PAI-1, while PAI-1-H/Kβ mice over-express the wild-type mouse sequence. There are potential differences in glycosylation, folding of the stabilised active form compared to the latent form and species-specific structures of the exogenous and endogenous proteins that may account for the apparent differences mucosal protection. In addition, PAI-1 may exhibit a U-shaped dose-response curve. This principle underpins the concept of adaptive cytoprotection, whereby exposure of the gastric mucosa to a low dose of a necrotising agent (mucosal irritant) or mild stress actually primes the cytoprotective mechanisms, so that the mucosa is protected against exposure to a more noxious dose of the same or another necrotising agent or severe stress.

Adaptive cytoprotection has been widely attributed to an increase in prostaglandin-mediated gastroprotective mechanisms *in vivo* and in isolated gastric cells (Konturek *et al.*, 1982; Robert *et al.*, 1983; Orihata *et al.*, 1989; Brzozowski *et al.*, 2000; Takeuchi *et al.*, 2001; Tsutsumi *et al.*, 2003). Given that increased expression of PAI-1 has been shown to protect against indomethacin-induced injury, the protection afforded by gastric PAI-1 must be independent of prostaglandin production. Some studies have reported that there is still an adaptive cytoprotective response following suppression of prostaglandin production with NSAIDs, so it may be that gastric PAI-1 elicits adaptive cytoprotection via alternative mechanisms, such as the formation of a protective layer of surface epithelial debris, modulation of surface-active phospholipid availability, heat shock protein induction, stimulation of mucus and/or bicarbonate production, by increasing NO production or by increasing the rate of epithelial cell restitution in response to mucosal injury (Hawkey *et al.*, 1988; Wallace, 1988; Nakamura *et al.*, 1991; Lugea *et al.*, 1994; Mutoh *et al.*, 1995; Konturek, 1997; Tanaka *et al.*, 2004; Gambero *et al.*, 2007). The precise nature of how PAI-1 might act as an irritant to the gastric mucosa, eliciting cytoprotection at low concentrations such as those produced by expression of both wild-type and transgenic PAI-1, but exacerbating indomethacin-induced injury at high exogenous concentrations, requires further investigation.

An alternative hypothesis for the opposing effects of gastric PAI-1 expression and exogenous PAI-1 administration involves the cytoprotection-mediating activity of the vagus nerve. Animals having undergone acute surgical vagotomy consistently developed more severe mucosal lesions in response to various necrotising agents, had impaired prostaglandin and glutathione production in response to a necrotising dose of ethanol and displayed a reversal of cytoprotection by various mediators, including prostaglandins, β-carotene, atropine and cimetidine (Mozsik *et al.*, 1991; Suto *et al.*, 1992; Karadi *et al.*, 1999; Karadi *et al.*, 2001; Mozsik *et al.*, 2001). An increase in vascular permeability in response to exposure to necrotising agents following vagotomy has been proposed to account, at least in part, for the more severe responses seen in these animals (Mozsik *et al.*, 1992; Karadi *et al.*, 1999; Karadi *et al.*, 2001). Furthermore, both endogenous and exogenous CCK, acting via CCK-1 receptors, have been shown to be gastroprotective via a mechanisms involving vagal capsaicin-sensitive afferent fibres and cholinergic efferent fibres and eliciting a local hyperaemic response mediated by CGRP and NO (Evangelista & Maggi, 1991; Konturek *et al.*, 1995; Heinemann *et al.*, 1996). Taken together, the results of these studies imply a crucial role for stimulation of the vago-vagal reflex by CCK in gastric cytoprotection. A previous study has shown that IP administration of PAI-1, at the same dose used in these experiments, inhibited the satiety effects of CCK and was associated with a reduction in the number of c-fos-expressing neurons in the nucleus tractus solitarius, a marker of vagal afferent neuronal activation (Kenny *et al.*, 2013a). This implies that IP administration of PAI-1 at this dose inhibits activation of the vago-vagal reflex by CCK, and may account for the more severe indomethacin-induced gastric mucosal injury observed after exogenous PAI-1. Studies might now be conducted in wild-type mice to examine the effects of CCK alone and co-administration of CCK and PAI-1 on the development of indomethacin-induced injury, to test this hypothesis.

CCK-stimulated vago-vagal reflexes are attenuated in PAI-1-H/Kβ mice (Kenny *et al.*, 2013a), so inhibition of this pathway by gastric PAI-1 seems at odds with the gastroprotection seen in these mice. Local gastroprotective activity of PAI-1 produced at the mucosa might have a net dominant effect over the inhibition of cytoprotection mediated by the vagus nerve. The potential contribution of gastric PAI-1 to gastroprotection via inhibition of extracellular plasmin generation has been discussed and may account for the balance towards cytoprotection in PAI-1-H/Kβ mice. Alternatively, it is possible that mucosal PAI-1 influences intracellular signalling via uPAR, favouring cytoprotective mechanisms such as enhanced epithelial cell proliferation and/or migration, suppression of extracellular protease synthesis by epithelial or stromal cells, suppression of hydrochloric acid secretion or enhanced secretion of mucus and/or bicarbonate. PAI-1-stimulated cytoprotective intracellular signalling pathways via uPAR could account for the more severe mucosal injury observed in uPAR$^{-/-}$ mice in response to intragastric ethanol (Kenny, 2008) and the tendency towards increased lesion development in response to indomethacin observed in this study.

The data presented in this chapter provides new insights into the role of the uPA system, particularly PAI-1, in gastric mucosal protection and responses to NSAID injury. This information has potential translational utility; in elucidating balance of expression of components of the uPA system that optimally protects the gastric mucosa against NSAID injury, therapeutic agents may be developed that prime the uPA system for gastroprotection. Such agents would reduce the risks associated with NSAID treatment, improving clinical outcomes for patients requiring chronic NSAID treatment.

6.5 Conclusions

1. Gastric PAI-1 is protective against indomethacin-induced mucosal injury.
2. Gastric PAI-1 may act via uPAR to protect the gastric mucosa against indomethacin-induced mucosal injury.
3. Exogenous PAI-1 exacerbates indomethacin-induced gastric mucosal injury.
4. Increased gastric uPA expression has no effect upon the development of indomethacin-induced mucosal injury.
5. Increases in corpus mucosal myofibroblast abundance become apparent between 5 and 7 days after injury induced by 20mg/kg intragastric indomethacin.

CHAPTER 7

DISCUSSION

7.1 Overview

The main findings of this thesis are: a) mice over-expressing PAI-1 in the gastric mucosa develop age-dependent increases in corpus mucosal thickness, independently of changes in parietal cell or myofibroblast abundance; b) mucosal hyperplasia in these mice is not due to a direct trophic action of PAI-1 on gastric epithelial cells; c) there is a highly heterogeneous population of myofibroblast-like cells in the mouse antral mucosa; d) mouse antral myofibroblasts in culture express markers of a neuroendocrine-like phenotype; e) both absence of PAI-1 and over-expression of gastric PAI-1 protect against mucosal responses to murine *H. felis* infection; f) absence of PAI-1 exacerbates the development of lesions in a murine model of acute gastric injury induced by intragastric indomethacin, whilst (g) expression of PAI-1 in the stomach of transgenic mice protects against indomethacin-induced injury; h) increased expression of gastric uPA has no effect upon indomethacin-induced injury, whilst i) mice lacking uPAR tend to develop more lesions.

7.2 Conceptual advances and future studies

The uPA system has well characterised roles in regulating tissue homeostasis, wound healing, fibrosis, inflammation, and cancer, via regulation of plasmin-mediated extracellular proteolysis as well its effects upon cell adhesion, migration and signalling (Andreasen *et al.*, 2000; Smith & Marshall, 2010). However, its role in gastric mucosal tissue homeostasis and responses to acute and chronic challenges has not been investigated. This work presented in this thesis contributes to the elucidation of the role of PAI-1 in acute responses to gastric mucosal injury and chronic *Helicobacter*-induced preneoplastic histopathology, as well as its role in shaping gastric corpus mucosal morphology in the absence of challenge. This thesis showed that PAI-1 is protective both in acute responses to NSAID-induced gastric injury, and in responses to chronic *H. felis* infection. Furthermore, absence of PAI-1 was also protective against *H. felis*-induced histopathogenesis. Increased gastric PAI-1 expression induced age-dependent increases in mucosal thickness, in the absence of challenge. However, the epithelial cell dynamics underlying these gastric mucosal phenotypes remains to be established.

The work presented in this thesis demonstrated that PAI-1 does not directly modulate proliferation of gastric epithelial cells. Previous work in this laboratory determined that PAI-1 suppresses *Helicobacter*-stimulated proliferation of gastric epithelial cells, via inhibition of uPA-mediated proteolytic release of HB-EGF (Kenny *et al.*, 2008). However, it is still not known how PAI-1 regulates proliferation *in vivo*. It might be hypothesised that PAI-1 stimulates proliferation *in vivo*, based on the observation that PAI-1-H/Kβ mice develop increased corpus mucosal thickness. This could be relevant to the role of PAI-1 in protection against gastric mucosal injury, a situation where accelerated epithelial restitution would be beneficial (Lacy & Ito, 1984; Konturek, 1990). Furthermore, absence of PAI-1 might inhibit *Helicobacter*-induced proliferation, a hallmark of early pre-neoplastic histopathogenesis (Lynch *et al.*, 1995; Fox *et al.*, 1996; McNamara & El-Omar, 2008), and may account in part for the protection seen in PAI-1$^{-/-}$ mice. In order to test these hypotheses, determination of *in vivo* epithelial cell proliferation in the gastric mucosa of unchallenged PAI-1-H/Kβ mice compared to wild-type and PAI-1$^{-/-}$ mice, in chronically *H. felis*-infected wild-type, PAI-1$^{-/-}$ and PAI-1-H/Kβ mucosa and in acute responses to indomethacin-induced injury of the gastric mucosa of wild-type, PAI-1$^{-/-}$ and PAI-1-H/Kβ mice is required. Incorporation of EdU into the gastrointestinal mucosa in live animals has been used as a marker of *in vivo* epithelial cell proliferation previously (Salic & Mitchison, 2008). Similar methods were used in this thesis, in pilot studies investigating whether pleiotrophin stimulates proliferation *in vivo*. This method could be expanded into the proposed studies.

Another important element of epithelial cell dynamics that could be relevant to the phenotypes reported in this thesis is apoptosis. PAI-1 may influence epithelial cell turnover via modulation of apoptosis in the gastric mucosa, contributing to increased mucosal thickness of PAI-1-H/Kβ mice. Similarly, modulation of apoptosis by PAI-1 may contribute to the resistance against injurious responses to acute and chronic gastric mucosal insult observed in PAI-1-H/Kβ mice. *Helicobacter*-stimulated preneoplastic histopathogenesis is associated with increased apoptosis of gastric epithelial cells (Moss *et al.*, 1996; Wang *et al.*, 1998; Court *et al.*, 2003). Similarly, acute gastric mucosal injury is associated with increases in epithelial apoptosis, and agents that prevent mucosal injury work in part by attenuating this increase in apoptosis (Piotrowski *et al.*, 1997; Slomiany *et al.*, 1997; Jones *et al.*, 2008; Hirata *et al.*, 2009). Based on the results presented in this thesis, it might be hypothesised that PAI-1 negatively regulates apoptosis, thus protecting the gastric mucosa. Since the basal rate of epithelial cell apoptosis is already very low in the gastric mucosa, a highly sensitive method would need to be employed to detect and quantify apoptotic cells. A qPCR-based method has been developed for the absolute quantification of apoptotic DNA in a population of cells, which might be used to compare total quantities of apoptotic cells in freshly isolated gastric epithelial cell populations from whole corpus tissues (Hooker *et al.*, 2012).

Another factor to consider in interpreting the phenotypes reported in PAI-1-H/Kβ mice is that these mice are likely to develop adaptive changes to persistent elevations in gastric PAI-1 expression, which might confound the analysis. In order to determine the effects of increased gastric PAI-1 in isolation on gastric mucosal responses to insult, models of inducible transgenic PAI-1 need to be developed. A commonly used technique for temporally controlled gene expression in transgenic mouse models is the tamoxifen-inducible Cre-loxP recombination system, which can be used to activate transgenes prenatally and postnatally (Hayashi & McMahon, 2002). Using this technology, expression of the gastric PAI-1 transgene could be induced immediately prior to administration of indomethacin. This model could also be used to determine the role of increased gastric PAI-1 in shaping the adult gastric mucosal phenotype during mucosal morphogenesis in the developing mouse stomach, by inducing the gastric PAI-1 transgene at various stages of embryonic and postnatal development. These studies may provide some insights into early influences on the development age-dependent increases in corpus mucosal thickness in PAI-1-H/Kβ mice.

One of the approaches used in this thesis to investigate how PAI-1 might modulate the mucosal microenvironment was to examine corpus mucosal myofibroblast abundance of wild-type, PAI-1$^{-/-}$ and PAI-1-H/Kβ mice. This was determined in the unchallenged gastric mucosa, in responses to *Helicobacter* infection and in responses to indomethacin-induced injury. The rationale for this was that myofibroblasts are important mediators of epithelial-mesenchymal interactions involved in regulating the gastrointestinal mucosal microenvironment (Valentich & Powell, 1994; Powell *et al.*, 1999; Wu *et al.*, 1999; Powell *et al.*, 2005; Powell *et al.*, 2011). Myofibroblast abundance was not altered in the absence of PAI-1 or by increased gastric PAI-1 expression under basal conditions, but in response to *Helicobacter* infection, both absence of PAI-1 and increased gastric PAI-1 expression protected against increased myofibroblast abundance. Attempts were also made to characterise changes in myofibroblast abundance in response to indomethacin treatment, but changes were only detectable beyond the ethical end-point, owing to mortality associated with responses to indomethacin. Therefore, alternative approaches are required to investigate gastric mucosal myofibroblast dynamics in response to indomethacin treatment, to provide further elucidation into the role of PAI-1 in regulating myofibroblast recruitment to the gastric mucosa.

One possible alternative approach could be based on long-term three-dimensional models of cultured gastric glands that have recently been developed, in which the stem cell niche is maintained by interactions between epithelial cells and myofibroblasts, for up to 3 months (Katano *et al.*, 2013). This is currently the best available model for recapitulating the gastric mucosal microenvironment *in vitro* and could potentially be informative in characterising myofibroblast responses to acute gastric epithelial injury. However, certain inherent limitations exist with this model, including loss of diversity of differentiated gastric epithelial cell populations, accelerated epithelial cell turnover and lack of stromal complexity compared to the intact gastric gland. Nevertheless, three-dimensional cultures of gastric glands from wild-type, PAI-1$^{-/-}$ and PAI-1-H/Kβ mice could be established, to compare features of the longer-term responses to epithelial injury, such as myofibroblast recruitment and epithelial dynamics.

Questions still remain as to how expression of PAI-1 in the gastric mucosa modulates the mucosal microenvironment, which may be relevant to the basal epithelial cell turnover, responses to *Helicobacter* infection and responses to indomethacin-induced injury. Secretomes of gastric epithelial cells and myofibroblasts, as well as other stromal cells, determine the gastric microenvironment, hence if PAI-1 were to modulate expression of secreted proteins, the microenvironment would be altered. In order to investigate the effects of PAI-1 in the gastric microenvironment on myofibroblast gene expression, particularly relating to secreted proteins, whole genome microarrays were undertaken on wild-type and PAI-1$^{-/-}$ gastric myofibroblasts. The rationale for this work was to identify differentially expressed transcripts of secreted proteins that might modulate gastric epithelial cell turnover, based on the observation that PAI-1-H/Kβ mice develop increased corpus mucosal thickness. As discussed in chapter 4, further work is required to identify such transcripts from the microarray data and test the functional significance of their secretion into the gastric mucosal microenvironment. Transcripts of particular interest include DPP4 and pleiotrophin, for which some initial functional studies were undertaken during the preparation of this thesis. Furthermore, the microarray data could be examined to identify differentially expressed transcripts that might be significant in protection against indomethacin-induced injury by gastric expression of PAI-1, or in protection against *Helicobacter*-induced histopathology.

A major limitation in interpreting the physiological relevance of the microarray data is that it is increasingly recognised that myofibroblasts are likely to adapt to culture conditions, deviating from their *in vivo* phenotypes. Furthermore, it is likely that heterogeneity of the gastric mucosal myofibroblast population *in vivo* is lost in culture owing to selective adaptation to the *in vitro* microenvironment. These phenomena, discussed in more detail in section 4.4, highlight the benefits of performing studies *in vivo*, or at least of developing *in vitro* models that recapitulate the complexity of epithelial-stromal interactions *in vivo*. Based on this reasoning, it might be preferable to use laser capture microdissection to select all myofibroblasts from intact tissue samples, in order to conduct gene expression studies in the future. This approach has been used successfully to characterise gene expression by isolated gastric CAFs, whole gastric cancer stroma, gastric cancer cells and specific gastric epithelial cell populations (Kazumori *et al.*, 2001; Wu *et al.*, 2005; Resnick *et al.*, 2006; Makino *et al.*, 2008; Sung *et al.*, 2011). This technique could be used to extract gastric mucosal myofibroblasts from *Helicobacter*-infected and indomethacin-treated mice, as well as from unchallenged mice, to identify differentially expressed transcripts that might be relevant to the phenotypes reported in this thesis.

A more direct measurement of altered secretomes from PAI-$1^{-/-}$ and PAI-1-H/Kβ myofibroblasts, compared to wild-type, might involve utilising proteomic techniques, such as isobaric tags for relative and absolute quantification or stable isotope labeling by amino acids in cell culture, on conditioned media from low passage cultured gastric myofibroblasts. These techniques have previously been used to compare the secretomes of gastric CAMs with those of ATMs (Holmberg *et al.*, 2012; Holmberg *et al.*, 2013). These techniques might also be used to characterise the secretomes of gastric epithelial cells and other gastric mucosal stromal cell populations. Information about the secretomes of gastric epithelial cells, myofibroblasts and other stromal cell populations might then be integrated to generate a model of the gastric mucosal microenvironment in the unchallenged mucosa, in responses to *Helicobacter* infection and responses to indomethacin-induced injury, and its modulation by gastric PAI-1 expression. Alternatively, proteomic analysis of conditioned media from three-dimensional gastric gland cultures might be utilised, as an integrated model of epithelial-myofibroblast interactions in the mucosal microenvironment.

The data presented in this thesis imply that myofibroblasts in the antral mucosa exist as a highly heterogeneous population. It is possible that phenotypically distinct myofibroblasts and myofibroblast-like cells differentially regulate the gastric mucosal microenvironment. Further phenotypic characterisation of the antral mucosal myofibroblast population might facilitate functional studies on these cells. Following identification of cell-specific markers, it might be possible in future to ablate individual populations of myofibroblasts and myofibroblast-like cells, using techniques similar to those used to target and ablate parietal cells and peritoneal fibroblasts (Li *et al.*, 1996; Okada *et al.*, 2003; Powell *et al.*, 2011). In this way, the functional significance of these myofibroblast sub-populations in the unchallenged gastric mucosa and in responses to *Helicobacter* and indomethacin might be determined.

It is important to consider that other stromal cell populations, as well as myofibroblasts, shape the gastric mucosal microenvironment. Paracrine secretions from inflammatory, immune and vascular cells, as well as endocrine and neural inputs, are key determinants of epithelial-stromal interactions maintaining basal mucosal morphology and responses to acute and chronic mucosal insults. With this in mind, future work might be directed towards investigating inflammatory responses during indomethacin-induced gastric mucosal injury in the mouse strains used in this thesis.

Pro-inflammatory cytokines and PGE2 stimulate expression of uPA and uPAR by gastric fibroblasts, whilst indomethacin suppresses their expression (Iwamoto *et al.*, 2003). Furthermore, expression of gastric PAI-1 is increased in biopsies from patients treated with NSAIDs compared to non-NSAID treated patients, and in response to indomethacin in mice (Kenny *et al.*, 2013b). This might imply a role for the uPA system in the acute inflammatory response to gastric injury, and may in part account for the deleterious effect of indomethacin on the gastric mucosa.

The molecular mechanisms that mediate protection by PAI-1 in responses to acute and chronic insult remain to be determined. PAI-1 is able to modulate molecular interactions via uPAR, independently of interactions with uPA, as well as via uPA-mediated molecular interactions (Andreasen *et al.*, 2000; Smith & Marshall, 2010). Some insight has been gained into the role of uPAR in mediating protection against mucosal injury. The data presented in this thesis showed that uPAR$^{-/-}$ mice tended to develop more extensive mucosal injury in response to indomethacin. In previous studies conducted in this laboratory, uPAR$^{-/-}$ mice developed significantly more severe gastric mucosal injury in response to ethanol (Kenny, 2008). These observations suggest that uPAR might mediate protective mechanisms induced by increased gastric expression of PAI-1. Interpreting the role of uPA in regulating the balance between gastric mucosal defence and injury is less straightforward; uPA-H/Kβ mice developed lesions with similar severity as wild-type mice in response to indomethacin, but developed significantly more severe injury in response to ethanol (Kenny, 2008). In order to gain similar insights into the role uPA- and uPAR-mediated pathways of PAI-1 activity in *Helicobacter* infection, chronic *H. felis* studies need to be conduced in uPAR$^{-/-}$ and uPA-H/Kβ mice. Given that gastric expression of both uPA and uPAR are elevated in response to

Helicobacter infection, increases in gastric PAI-1 expression might function to counteract deleterious activities of these proteins, such as proliferation via EGFR (Kenny *et al.*, 2008).

Certain point mutations in the PAI-1 gene are known to render the protein deficient in vitronectin binding activity (PAI-1^{Q123K} or PAI-1^{Q123K,R101A}), LRP-binding activity (PAI-1^{R76E}) or tPA/uPA-inhibiting activity (PAI-1^{R346M,M347S} or PAI-1^{R346A}) (Shubeita *et al.*, 1990; Lawrence *et al.*, 1994; Stefansson *et al.*, 1998; Xu *et al.*, 2004). Utilising the same methodology used to generate PAI-1$^{-/-,TG+}$ mice (see section 2.2.2), mice expressing these PAI-1 mutant isoforms in the gastric mucosa could be generated, free of systemic and gastric wild-type PAI-1 expression. A similar strategy has been used to generate transgenic mice expressing PAI-1 mutant isoforms under the control of the murine preproendothelin-1 promoter (Eren *et al.*, 2007). Responses to chronic *H. felis* infection and to indomethacin-induced injury by mice expressing mutated PAI-1, compared to PAI-1$^{-/-,TG+}$ mice, might elucidate the relative contributions of proteolytic and non-proteolytic pathways of PAI-1 regulatory activities in histopathological responses to *Helicobacter* infection and in responses to indomethacin.

Some of the results presented in this thesis support previous studies reporting the paradoxical nature of PAI-1 biology, which renders its role in any tissue microenvironment highly dependent upon its bioavailability. In this thesis, both absence of PAI-1 and increased PAI-1 protected against *Helicobacter*-induced gastric histopathology. Whilst endogenous gastric PAI-1 expression had no effect upon IGF-II stimulated proliferation of gastric epithelial cells, exogenous PAI-1 suppressed its effects. Furthermore, contrary to the protective effects of gastric

PAI-1, exogenous PAI-1 exacerbated lesion development in response to indomethacin. However, many factors need to be considered when interpreting the results of experiments using exogenous PAI-1. Given the complex biology of PAI-1, deviations from the molecular structure of endogenous PAI-1, such as glycosylation, stability and species-specific protein structure, might have profound effects upon the molecular interactions of exogenous PAI-1 within the gastric mucosal microenvironment, so that it may not represent the physiological activities of endogenous PAI-1 in the gastric mucosa. This again highlights the benefits of developing robust *in vivo* models of inducible PAI-1 expression, to investigate the role of PAI-1 in regulating the gastric mucosal microenvironment and determining gastric mucosal responses.

Taken together, the data presented in this thesis deliver evidence of a broadly protective role of PAI-1 in the gastric mucosa. Although the mechanisms through which PAI-1 modulates the gastric mucosal microenvironment to endow protection against acute and chronic insults may be distinct from each other, a common therapeutic strategy for the prevention of NSAID-induced gastric injury, ulcer complications and progression of *Helicobacter*-induced gastric preneoplasia might be emerging, aimed at specifically increasing PAI-1 bioavailability in the gastric mucosa.

REFERENCES

Abe S, Sasano H, Katoh K, Ohara S, Arikawa T, Noguchi T, Asaki S, Yasui W, Tahara E, Nagura H & Toyota T (1997). Immunohistochemical studies on EGF family growth factors in normal and ulcerated human gastric mucosa. *Dig Dis Sci* **42,** 1199-1209.

Abraham E, Gyetko MR, Kuhn K, Arcaroli J, Strassheim D, Park JS, Shetty S & Idell S (2003). Urokinase-type plasminogen activator potentiates lipopolysaccharide-induced neutrophil activation. *J Immunol* **170,** 5644-5651.

Adegboyega PA, Mifflin RC, DiMari JF, Saada JI & Powell DW (2002). Immunohistochemical study of myofibroblasts in normal colonic mucosa, hyperplastic polyps, and adenomatous colorectal polyps. *Arch Pathol Lab Med* **126,** 829-836.

Aderem A & Ulevitch RJ (2000). Toll-like receptors in the induction of the innate immune response. *Nature* **406,** 782-787.

Aguirre Ghiso JA, Kovalski K & Ossowski L (1999). Tumor dormancy induced by downregulation of urokinase receptor in human carcinoma involves integrin and MAPK signaling. *J Cell Biol* **147,** 89-104.

Aguirre Ghiso JA (2002). Inhibition of FAK signaling activated by urokinase receptor induces dormancy in human carcinoma cells in vivo. *Oncogene* **21,** 2513-2524.

Aihara T, Fujishita T, Kanatani K, Furutani K, Nakamura E, Taketo MM, Matsui M, Chen D & Okabe S (2003). Impaired gastric secretion and lack of trophic responses to hypergastrinemia in M3 muscarinic receptor knockout mice. *Gastroenterology* **125,** 1774-1784.

Akita S, Kubota K, Kobayashi A, Misawa R, Shimizu A, Nakata T, Yokoyama T, Takahashi M & Miyagawa S (2012). Role of bone marrow cells in the development of pancreatic fibrosis in a rat model of pancreatitis induced by a choline-deficient/ethionine-supplemented diet. *Biochem Biophys Res Commun* **420,** 743-749.

Al-Fakhri N, Chavakis T, Schmidt-Wöll T, Huang B, Cherian SM, Bobryshev YV, Lord RS, Katz N & Preissner KT (2003). Induction of apoptosis in vascular cells by plasminogen activator inhibitor-1 and high molecular weight kininogen correlates with their anti-adhesive properties. *Biol Chem* **384,** 423-435.

Al-Jiboury H & Kaunitz JD (2012). Gastroduodenal mucosal defense. *Curr Opin Gastroenterol.* **28,** 594-601.

Alfano D, Iaccarino I & Stoppelli MP (2006). Urokinase signaling through its receptor protects against anoikis by increasing BCL-xL expression levels. *J Biol Chem* **281,** 17758-17767.

Allen A & Flemström G (2005). Gastroduodenal mucus bicarbonate barrier: protection against acid and pepsin. *Am J Physiol Cell Physiol* **288,** C1-19.

Andoh A, Bamba S, Brittan M, Fujiyama Y & Wright NA (2007). Role of intestinal subepithelial myofibroblasts in inflammation and regenerative response in the gut. *Pharmacol Ther* **114,** 94-106.

Andreasen PA, Riccio A, Welinder KG, Douglas R, Sartorio R, Nielsen LS, Oppenheimer C, Blasi F & Danø K (1986). Plasminogen activator inhibitor type-1: reactive center and amino-terminal heterogeneity determined by protein and cDNA sequencing. *FEBS Lett* **209,** 213-217.

Andreasen PA, Egelund R & Petersen HH (2000). The plasminogen activation system in tumor growth, invasion, and metastasis. *Cell Mol Life Sci* **57,** 25-40.

Aoyagi-Ikeda K, Maeno T, Matsui H, Ueno M, Hara K, Aoki Y, Aoki F, Shimizu T, Doi H, Kawai-Kowase K, Iso T, Suga T, Arai M & Kurabayashi M (2011). Notch induces myofibroblast differentiation of alveolar epithelial cells via transforming growth factor-β-Smad3 pathway. *Am J Respir Cell Mol Biol* **45,** 136-144.

Ardite E, Perdiguero E, Vidal B, Gutarra S, Serrano AL & Muñoz-Cánoves P (2012). PAI-1-regulated miR-21 defines a novel age-associated fibrogenic pathway in muscular dystrophy. *J Cell Biol* **196,** 163-175.

Ashcroft FJ, Varro A, Dimaline R & Dockray GJ (2004). Control of expression of the lectin-like protein Reg-1 by gastrin: role of the Rho family GTPase RhoA and a C-rich promoter element. *Biochem J* **381,** 397-403.

Ashurst HL, Varro A & Dimaline R (2008). Regulation of mammalian gastrin/CCK receptor (CCK2R) expression in vitro and in vivo. *Exp Physiol* **93,** 223-236.

Atherton JC, Peek RM Jr, Tham KT, Cover TL & Blaser MJ (1997). Clinical and pathological importance of heterogeneity in vacA, the vacuolating cytotoxin gene of Helicobacter pylori. *Gastroenterology* **112,** 92-99.

Atuma C, Strugala V, Allen A & Holm L (2001). The adherent gastrointestinal mucus gel layer: thickness and physical state in vivo. *Am J Physiol Gastrointest Liver Physiol* **280,** G922-G929.

Azuma T, Suto H, Ito Y, Ohtani M, Dojo M, Kuriyama M & Kato T (2001). Gastric leptin and Helicobacter pylori infection. *Gut* **49,** 324-329.

Backert S, Ziska E, Brinkmann V, Zimny-Arndt U, Fauconnier A, Jungblut PR, Naumann M & Meyer TF (2000). Translocation of the Helicobacter pylori CagA protein in gastric epithelial cells by a type IV secretion apparatus. *Cell Microbiol* **2,** 155-164.

Bado A, Levasseur S, Attoub S, Kermorgant S, Laigneau JP, Bortoluzzi MN, Moizo L, Lehy T, Guerre-Millo M, Le Marchand-Brustel Y & Lewin MJ (1998). The stomach is a source of leptin. *Nature* **394,** 790-793.

Baek MK, Kim MH, Jang HJ, Park JS, Chung IJ, Shin BA, Ahn BW & Jung YD (2008). EGF stimulates uPAR expression and cell invasiveness through ERK, AP-1, and NF-kappaB signaling in human gastric carcinoma cells. *Oncol Rep* **20,** 1569-1575.

Bajou K, Noël A, Gerard RD, Masson V, Brunner N, Holst-Hansen C, Skobe M, Fusenig NE, Carmeliet P, Collen D & Foidart JM (1998). Absence of host plasminogen activator inhibitor 1 prevents cancer invasion and vascularization. *Nat Med* **4,** 923-928.

Bajou K, Maillard C, Jost M, Lijnen RH, Gils A, Declerck P, Carmeliet P, Foidart JM & Noel A (2004). Host-derived plasminogen activator inhibitor-1 (PAI-1) concentration is critical for in vivo tumoral angiogenesis and growth. *Oncogene* **23,** 6986-6990.

Balabanova S (2012). The neuroendocrine-like phenotype of gastric myofibroblasts and its significance in cancer. *PhD thesis, University of Liverpool, UK.*

Balsara RD, Castellino FJ & Ploplis VA (2006). A novel function of plasminogen activator inhibitor-1 in modulation of the AKT pathway in wild-type and plasminogen activator inhibitor-1-deficient endothelial cells. *J Biol Chem* **281,** 22527-22536.

Bamford KB, Fan X, Crowe SE, Leary JF, Gourley WK, Luthra GK, Brooks EG, Graham DY, Reyes VE & Ernst PB (1998). Lymphocytes in the human gastric mucosa during Helicobacter pylori have a T helper cell 1 phenotype. *Gastroenterology* **114,** 482-492.

Baragi VM, Qiu L, Gunja-Smith Z, Woessner JF Jr, Lesch CA & Guglietta A (1997). Role of metalloproteinases in the development and healing of acetic acid-induced gastric ulcer in rats. *Scand J Gastroenterol* **32,** 419-426.

Barker N, Huch M, Kujala P, van de Wetering M, Snippert HJ, van Es JH, Sato T, Stange DE, Begthel H, van den Born M, Danenberg E, van den Brink S, Korving J, Abo A, Peters PJ, Wright N, Poulsom R & Clevers H (2010). Lgr5(+ve) stem cells drive self-renewal in the stomach and build long-lived gastric units in vitro. *Cell Stem Cell* **6,** 25-36.

Barrachina MD, Martínez V, Wang L, Wei JY & Taché Y (1997). Synergistic interaction between leptin and cholecystokinin to reduce short-term food intake in lean mice. *Proc Natl Acad Sci U S A* **S94,** 10455-10460.

Basso D, Navaglia F, Brigato L, Piva MG, Toma A, Greco E, Di Mario F, Galeotti F, Roveroni G, Corsini A & Plebani M (1998). Analysis of Helicobacter pylori vacA and cagA genotypes and serum antibody profile in benign and malignant gastroduodenal diseases. *Gut* **43,** 182-186.

Bauman KA, Wettlaufer SH, Okunishi K, Vannella KM, Stoolman JS, Huang SK, Courey AJ, White ES, Hogaboam CM, Simon RH, Toews GB, Sisson TH, Moore BB & Peters-Golden M (2010). The antifibrotic effects of plasminogen activation occur via prostaglandin E2 synthesis in humans and mice. *Clin Invest* **120,** 1950-1960.

Bayliss WM & Starling EH (1902). The mechanism of pancreatic secretion. *J Physiol* **28,** 325-353.

Berglindh T & Obrink KJ (1976). A method for preparing isolated glands from the rabbit gastric mucosa. *Acta Physiol Scand* **96,** 150-159.

Berthoud HR & Powley TL (1992). Vagal afferent innervation of the rat fundic stomach: morphological characterization of the gastric tension receptor. *J Comp Neurol* **319,** 261-276.

Beyer BC, Heiss MM, Simon EH, Gruetzner KU, Babic R, Jauch KW, Schildberg FW & Allgayer H (2006). Urokinase system expression in gastric carcinoma: Prognostic impact in an independent patient series and first evidence of predictive value in preoperative biopsy and intestinal metaplasia specimens. *Cancer* **106,** 1026-1035.

Bianchi E, Ferrero E, Fazioli F, Mangili F, Wang J, Bender JR, Blasi F & Pardi R (1996). Integrin-dependent induction of functional urokinase receptors in primary T lymphocytes. *J Clin Invest* **98,** 1133-1141.

Binai N, O'Reilly S, Griffiths B, van Laar JM & Hügle T (2012). Differentiation potential of CD14+ monocytes into myofibroblasts in patients with systemic sclerosis. *PLoS One* **7,** e33508.

Binder BR, Mihaly J & Prager GW (2007). uPAR-uPA-PAI-1 interactions and signaling: A vascular biologist’s view. *Thromb Haemost* **97,** 336-342.

Bindu S, Pal C, Dey S, Goyal M, Alam A, Iqbal MS, Dutta S, Sarkar S, Kumar R, Maity P & Bandyopadhyay U (2011). Translocation of heme oxygenase-1 to mitochondria is a novel cytoprotective mechanism against non-steroidal anti-inflammatory drug-induced mitochondrial oxidative stress, apoptosis, and gastric mucosal injury. *J Biol Chem* **286,** 39387-39402.

Birgersdotter A, Sandberg R & Ernberg I (2005). Gene expression perturbation in vitro--a growing case for three-dimensional (3D) culture systems. *Semin Cancer Biol* **15,** 405-412.

Bjerknes M & Cheng H (2002). Multipotential stem cells in adult mouse gastric epithelium. *Am J Physiol Gastrointest Liver Physiol* **283,** G767-G777.

Blaser MJ, Perez-Perez GI, Kleanthous H, Cover TL, Peek RM, Chyou PH, Stemmermann GN & Nomura A (1995). Infection with Helicobacter pylori strains possessing cagA is associated with an increased risk of developing adenocarcinoma of the stomach. *Cancer Res* **55,** 2111-2115.

Blasi F & Carmeliet P (2002). uPAR: a versatile signalling orchestrator. *Nat Rev Mol Cell Biol* **3,** 932-943.

Braaten JV, Handt S, Jerome WG, Kirkpatrick J, Lewis JC & Hantgan RR (1993). Regulation of fibrinolysis by platelet-released plasminogen activator inhibitor 1: light scattering and ultrastructural examination of lysis of a model platelet-fibrin thrombus. *Blood* **81,** 1290-1299.

Breidert M, Miehlke S, Glasow A, Orban Z, Stolte M, Ehninger G, Bayerdörffer E, Nettesheim O, Halm U, Haidan A & Bornstein SR (1999). Leptin and its receptor in normal human gastric mucosa and in Helicobacter pylori-associated gastritis. *Scand J Gastroenterol* **34,** 954-961.

Brzozowska I, Ptak-Belowska A, Pawlik M, Pajdo R, Drozdowicz D, Konturek SJ, Pawlik WW & Brzozowski T (2009). Mucosal strengthening activity of central and peripheral melatonin in the mechanism of gastric defense. *J Physiol Pharmacol* **60 Suppl 7,** 47-56.

Brzozowski T, Konturek PC, Konturek SJ, Drozdowicz D, Pajdo R, Pawlik M, Brzozowska I & Hahn EG (2000). Expression of cyclooxygenase (COX)-1 and COX-2 in adaptive cytoprotection induced by mild stress. *J Physiol Paris* **94,** 83-91.

Brzozowski T, Konturek PC, Pajdo R, Kwiecień S, Ptak A, Sliwowski Z, Drozdowicz D, Pawlik M, Konturek SJ & Hahn EG (2001). Brain-gut axis in gastroprotection by leptin and cholecystokinin against ischemia-reperfusion induced gastric lesions. *J Physiol Pharmacol* **52,** 583-602.

Brzozowski T, Konturek PC, Drozdowicz D, Konturek SJ, Pawlik M, Sliwowski Z, Pawlik WW & Hahn EG (2005). Role of central and peripheral ghrelin in the mechanism of gastric mucosal defence. *Inflammopharmacology* **13,** 45-62.

Brzozowski T, Konturek PC, Sliwowski Z, Drozdowicz D, Burnat G, Pajdo R, Pawlik M, Bielanski W, Kato I, Kuwahara A, Konturek SJ & Pawlik WW (2008). Gastroprotective action of orexin-A against stress-induced gastric damage is mediated by endogenous prostaglandins, sensory afferent neuropeptides and nitric oxide. *Regul Pept* **148,** 6-20.

Budinger GR, McKell JL, Urich D, Foiles N, Weiss I, Chiarella SE, Gonzalez A, Soberanes S, Ghio AJ, Nigdelioglu R, Mutlu EA, Radigan KA, Green D, Kwaan HC & Mutlu GM (2011). Particulate matter-induced lung inflammation increases systemic levels of PAI-1 and activates coagulation through distinct mechanisms. *PLoS One* **6,** e18525.

Bugge TH, Suh TT, Flick MJ, Daugherty CC, Romer J, Solberg H, Ellis V, Dano K & Degen JL (1995). The receptor for urokinase-type plasminogen activator is not essential for mouse development or fertility. *J Biol Chem* **270,** 16886-16894.

Cai X, Carlson J, Stoicov C, Li H, Wang TC & Houghton J (2005). Helicobacter felis eradication restores normal architecture and inhibits gastric cancer progression in C57BL/6 mice. *Gastroenterology* **128,** 1937-1952.

Campbell PG, Novak JF, Yanosick TB & McMaster JH (1992). Involvement of the plasmin system in dissociation of the insulin-like growth factor binding protein complex. *Endocrinology* **130,** 1401-1412.

Carmeliet P, Kieckens L, Schoonjans L, Ream B, van Nuffelen A, Prendergast G, Cole M, Bronson R, Collen D & Mulligan RC (1993). Plasminogen activator inhibitor-1 gene-deficient mice. I. Generation by homologous recombination and characterization. *J Clin Invest* **92,** 2746-2755.

Carmeliet P, Moons L, Lijnen R, Baes M, Lemaître V, Tipping P, Drew A, Eeckhout Y, Shapiro S, Lupu F & Collen D (1997). Urokinase-generated plasmin activates matrix metalloproteinases during aneurysm formation. *Nat Genet* **17,** 439-444.

Carson JL, Strom BL, Soper KA, West SL & Morse ML (1987). The association of nonsteroidal anti-inflammatory drugs with upper gastrointestinal tract bleeding. *Arch Intern Med* **147,** 85-88.

Carthy JM, Garmaroudi FS, Luo Z & McManus BM (2011). Wnt3a induces myofibroblast differentiation by upregulating TGF-β signaling through SMAD2 in a β-catenin-dependent manner. *PLoS One* **6,** e19809.

Castellino FJ & Ploplis VA (2005). Structure and function of the plasminogen/plasmin system. *Thromb Haemost* **93,** 647-654.

Chapman HA & Wei Y (2001). Protease crosstalk with integrins: the urokinase receptor paradigm. *Thromb Haemost* **86,** 124-129.

Chaturvedi R, Asim M, Romero-Gallo J, Barry DP, Hoge S, de Sablet T, Delgado AG, Wroblewski LE, Piazuelo MB, Yan F, Israel DA, Casero RA Jr, Correa P, Gobert AP, Polk DB, Peek RM Jr & Wilson KT (2011). Spermine oxidase mediates the gastric cancer risk associated with Helicobacter pylori CagA. *Gastroenterology* **141,** 1696-1708.

Chatzaki E, Lambropoulou M, Constantinidis TC, Papadopoulos N, Taché Y, Minopoulos G & Grigoriadis DE (2006). Corticotropin-releasing factor (CRF) receptor type 2 in the human stomach: protective biological role by inhibition of apoptosis. *J Cell Physiol* **209,** 905-911.

Chen HC, Zhu YT, Chen SY & Tseng SC (2012). Wnt signaling induces epithelial-mesenchymal transition with proliferation in ARPE-19 cells upon loss of contact inhibition. *Lab Invest* **92,** 676-687.

Chen SC, Henry DO, Reczek PR & Wong MK (2008). Plasminogen activator inhibitor-1 inhibits prostate tumor growth through endothelial apoptosis. *Mol Cancer Ther* **7,** 1227-1236.

Chen Y, Kelm RJ Jr, Budd RC, Sobel BE & Schneider DJ (2004). Inhibition of apoptosis and caspase-3 in vascular smooth muscle cells by plasminogen activator inhibitor type-1. *J Cell Biochem* **92,** 178-188.

Chen Y, Budd RC, Kelm RJ Jr, Sobel BE & Schneider DJ (2006). Augmentation of proliferation of vascular smooth muscle cells by plasminogen activator inhibitor type 1. *Arterioscler Thromb Vasc Biol* **26,** 1777-1783.

Choi YK, Yoon BI, Kook YH, Won YS, Kim JH, Lee CH, Hyun BH, Oh GT, Sipley J & Kim DY (2002). Overexpression of urokinase-type plasminogen activator in human gastric cancer cell line (AGS) induces tumorigenicity in severe combined immunodeficient mice. *Jpn J Cancer Res* **93,** 151-156.

Chorostowska-Wynimko J, Kedzior M, Struniawski R, Jaguś P, Skrzypczak-Jankun E & Jankun J (2010). Cell phenotype determines PAI-1 antiproliferative effect - suppressed proliferation of the lung cancer but not prostate cancer cells. *Pneumonol Alergol Pol* **78,** 279-283.

Choudhuri R, Zhang HT, Donnini S, Ziche M & Bicknell R (1997). An angiogenic role for the neurokines midkine and pleiotrophin in tumorigenesis. *Cancer Res* **57,** 1814-1819.

Conigrave AD, Quinn SJ & Brown EM (2000). L-amino acid sensing by the extracellular Ca2+-sensing receptor. *Proc Natl Acad Sci U S A* **97,** 4814-4819.

Correa P (1992). Human gastric carcinogenesis: a multistep and multifactorial process--First American Cancer Society Award Lecture on Cancer Epidemiology and Prevention. *Cancer Res* **52,** 6735-6740.

Courty J, Dauchel MC, Caruelle D, Perderiset M & Barritault D (1991). Mitogenic properties of a new endothelial cell growth factor related to pleiotrophin. *Biochem Biophys Res Commun* **180,** 145-151.

Cosgrove D (2007). A complex, tissue-specific role for plasmin and its regulators in modulating fibrogenic activity. *Am J Physiol Renal Physiol* **293,** F10-F11.

Court M, Robinson PA, Dixon MF & Crabtree JE (2002). Gastric *Helicobacter* species infection in murine and gerbil models: comparative analysis of effects of *H. pylori* and *H. felis* on gastric epithelial cell proliferation. *J Infect Dis* **186,** 1348-1352

Court M, Robinson PA, Dixon MF, Jeremy AHT & Crabtree JE (2003). The effect of gender on *Helicobacter felis*-mediated gastritis, epithelial cell proliferation, and apoptosis in the mouse model. *J Pathol* **201,** 303-311.

Cover TL & Blanke SR (2005). Helicobacter pylori VacA, a paradigm for toxin multifunctionality. *Nat Rev Microbiol* **3,** 320-332.

Cover TL & Blaser MJ (2009). Helicobacter pylori in health and disease. *Gastroenterology* **136,** 1863-1873.

Cox HT, Poller L & Thomson JM (1967). Gastric fibrinolysis. A possible aetiological link with peptic ulcer. *Lancet* **1,** 1300-1302.

Cox JM, Clayton CL, Tomita T, Wallace DM, Robinson PA & Crabtree JE (2001). cDNA array analysis of cag pathogenicity island-associated Helicobacter pylori epithelial cell response genes. *Infect Immun* **69,** 6970-6980.

Crabtree JE, Covacci A, Farmery SM, Xiang Z, Tompkins DS, Perry S, Lindley IJ & Rappuoli R (1995). Helicobacter pylori induced interleukin-8 expression in gastric epithelial cells is associated with CagA positive phenotype. *J Clin Pathol* **48,** 41-45.

Crabtree JE (1998). Role of cytokines in pathogenesis of Helicobacter pylori-induced mucosal damage. *Dig Dis Sci* **43,** 46S-55S.

Crabtree JE, Ferrero RL & Kusters JG (2002). The mouse colonizing Helicobacter pylori strain SS1 may lack a functional cag pathogenicity island. *Helicobacter* **7,** 139-140.

Crean GP, Marshall MW & Rumsey RD (1969). Parietal cell hyperplasia induced by the administration of pentagastrin (ICI 50,123) to rats. *Gastroenterology* **57,** 147-155.

Cui G, Koh TJ, Chen D, Zhao CM, Takaishi S, Dockray GJ, Varro A, Rogers AB, Fox JG & Wang TC (2004). Overexpression of glycine-extended gastrin inhibits parietal cell loss and atrophy in the mouse stomach. *Cancer Res* **64,** 8160-8166.

Cui G, Takaishi S, Ai W, Betz KS, Florholmen J, Koh TJ, Houghton J, Pritchard DM & Wang TC (2006). Gastrin-induced apoptosis contributes to carcinogenesis in the stomach. *Lab Invest* **86,** 1037-1051.

Cunliffe RN & Mahida YR (2004). Expression and regulation of antimicrobial peptides in the gastrointestinal tract. *J Leukoc Biol* **75,** 49-58.

Czekay RP, Aertgeerts K, Curriden SA & Loskutoff DJ (2003). Plasminogen activator inhibitor-1 detaches cells from extracellular matrices by inactivating integrins. *J Cell Biol* **160,** 781-791.

Dairkee SH, Ji Y, Ben Y, Moore DH, Meng Z & Jeffrey SS (2004). A molecular 'signature' of primary breast cancer cultures; patterns resembling tumor tissue. *BMC Genomics* **5,** 47.

Dass K, Ahmad A, Azmi AS, Sarkar SH & Sarkar FH (2008). Evolving role of uPA/uPAR system in human cancers. *Cancer Treatment Rev* **34,** 122-136.

Date Y, Kojima M, Hosoda H, Sawaguchi A, Mondal MS, Suganuma T, Matsukura S, Kangawa K & Nakazato M (2000). Ghrelin, a novel growth hormone-releasing acylated peptide, is synthesized in a distinct endocrine cell type in the gastrointestinal tracts of rats and humans. *Endocrinology* **141,** 4255-4261.

de Paulis A, Montuori N, Prevete N, Fiorentino I, Rossi FW, Visconte V, Rossi G, Marone G & Ragno P (2004). Urokinase induces basophil chemotaxis through a urokinase receptor epitope that is an endogenous ligand for formyl peptide receptor-like 1 and -like 2. *J Immunol* **173,** 5739-5748.

De Wever O, Demetter P, Mareel M & Bracke M (2008). Stromal myofibroblasts are drivers of invasive cancer growth. *Int J Cancer* **123,** 2229-2238.

Degryse B, Resnati M, Czekay RP, Loskutoff DJ & Blasi F (2005). Domain 2 of the urokinase receptor contains an integrin-interacting epitope with intrinsic signaling activity: generation of a new integrin inhibitor. *J Biol Chem* **280,** 24792-24803.

Del Rosso M, Margheri F, Serratì S, Chillà A, Laurenzana A & Fibbi G (2011). The urokinase receptor system, a key regulator at the intersection between inflammation, immunity, and coagulation. *Curr Pharm Des* **17,** 1924-1943.

Dembinski AB & Johnson LR (1979). Growth of pancreas and gastrointestinal mucosa in antrectomized and gastrin-treated rats. *Endocrinology* **105,** 769-773.

Deng G, Curriden S, Hu G, Czekay R & Loskutoff D (2001). Plasminogen activator inhibitor-1 regulates cell adhesion by binding to the somatomedin B domain of vitronectin. *J Cell Physiol* **189,** 23-33.

Deryugina EI & Quigley JP (2012). Cell surface remodeling by plasmin: a new function for an old enzyme. *J Biomed Biotechnol* **2012,** 564259.

Desmoulière A, Geinoz A, Gabbiani F & Gabbiani G (1993). Transforming growth factor-beta 1 induces alpha-smooth muscle actin expression in granulation tissue myofibroblasts and in quiescent and growing cultured fibroblasts. *J Cell Biol* **122,** 103-111.

Devy L, Blacher S, Grignet-Debrus C, Bajou K, Masson V, Gerard RD, Gils A, Carmeliet G, Carmeliet P, Declerck PJ, Noel A & Foidart JM (2002). The pro- or antiangiogenic effect of plasminogen activator inhibitor 1 is dose dependent. *FASEB J* **16,** 147-154.

Di Y, Liu Z, Tian J, Zong Y, Yang P & Qu S (2010). TFPI or uPA-PAI-1 complex affect cell function through expression variation of type II very low density lipoprotein receptor. *FEBS Lett* **584,** 3469-3473.

Dial EJ, Hall LR, Romero JJ, Lechago J, Fox JG & Lichtenberger LM (2000). Altered gastrin regulation in mice infected with Helicobacter felis. *Dig Dis Sci* **45,** 1308-1314.

Dickson JH, Grabowska A, El-Zaatari M, Atherton J & Watson SA (2006). Helicobacter pylori can induce heparin-binding epidermal growth factor expression via gastrin and its receptor. *Cancer Res* **66,** 7524-7531.

Diéval J, Nguyen G, Gross S, Delobel J & Kruithof EK (1991). A lifelong bleeding disorder associated with a deficiency of plasminogen activator inhibitor type 1. *Blood* **77,** 528-532.

Ding Y, Zhang H, Zhong M, Zhou Z, Zhuang Z, Yin H, Wang X & Zhu Z (2013). Clinical significance of the uPA system in gastric cancer with peritoneal metastasis. *Eur J Med Res* **18,** 28.

Djahanguiri B (1969). The production of acute gastric ulceration by indomethacin in the rat. *Scand J Gastroenterol* **4,** 265-267.

Dockray G (2003). Making sense of gut contents. *Scand J Gastroenterol* **38,** 451-455.

Du Y, Agnew A, Ye XP, Robinson PA, Forman D & Crabtree JE (2006). Helicobacter pylori and Schistosoma japonicum co-infection in a Chinese population: helminth infection alters humoral responses to H. pylori and serum pepsinogen I/II ratio. *Microbes Infect* **8,** 52-60.

Duckworth CA, Clyde D & Pritchard DM (2012). CD24 is expressed in gastric parietal cells and regulates apoptosis and the response to Helicobacter felis infection in the murine stomach. *Am J Physiol Gastrointest Liver Physiol* **303,** G915-G926.

Edkins JS (1905). On the chemical mechanism of gastric secretion. *Proc Roy Soc B* **76,** 376.

Edkins JS (1906). The chemical mechanism of gastric secretion, *J Physiol* **34,** 133-144.

Egelund R, Rodenburg KW, Andreasen PA, Rasmussen MS, Guldberg RE & Petersen TE (1998). An ester bond linking a fragment of a serine proteinase to its serpin inhibitor. *Biochem* **37,** 6375-6379.

El-Omar EM, Oien K, El-Nujumi A, Gillen D, Wirz A, Dahill S, Williams C, Ardill JE & McColl KE (1997). Helicobacter pylori infection and chronic gastric acid hyposecretion. *Gastroenterology* **113,** 15-24.

Emond M, Schwartz GJ, Ladenheim EE & Moran TH (1999). Central leptin modulates behavioral and neural responsivity to CCK. *Am J Physiol Regul Integr Comp Physiol* **276,** R1545-R1549.

Eren M, Gleaves LA, Atkinson JB, King LE, Declerck PJ & Vaughan DE (2007). Reactive site-dependent phenotypic alterations in plasminogen activator inhibitor-1 transgenic mice. *J Thromb Haemost* **5,** 1500-1508.

Ernst PB & Gold BD (2000). The disease spectrum of Helicobacter pylori: the immunopathogenesis of gastroduodenal ulcer and gastric cancer. *Annu Rev Microbiol* **54,** 615-640.

Estreicher A, Muhlhauser J, Carpentier JL, Orci L & Vassalli JD (1990). The receptor for urokinase type plasminogen activator polarizes expression of the protease to the leading edge of migrating monocytes and promotes degradation of enzyme inhibitor complexes. *J Cell Biol* **111,** 783-792.

Evangelista S & Maggi CA (1991). Protection induced by cholecystokinin-8 (CCK-8) in ethanol-induced gastric lesions is mediated via vagal capsaicin-sensitive fibers and CCK receptors. *Br J Pharmacol* **102,** 119-122.

Evans RA, Tian YC, Steadman R & Phillips AO (2003). TGF-beta1-mediated fibroblast-myofibroblast terminal differentiation-the role of Smad proteins. *Exp Cell Res* **282,** 90-100.

Eyden B (2008). The myofibroblast: phenotypic characterization as a prerequisite to understanding its functions in translational medicine. *J Cell Mol Med* **12**, 22-37.

Fang W, Hartmann N, Chow DT, Riegel AT & Wellstein A (1992). Pleiotrophin stimulates fibroblasts and endothelial and epithelial cells and is expressed in human cancer. *J Biol Chem* **267,** 25889-25897.

Farinati F, Herszényi L, Plebani M, Carraro P, De Paoli M, Cardin R, Roveroni G, Rugge M, Nitti D, Grigioni WF, D'Errico A & Naccarato R (1996). Increased levels of cathepsin B and L, urokinase-type plasminogen activator and its inhibitor type-1 as an early event in gastric carcinogenesis. *Carcinogenesis* **17,** 2581-2587.

Farrell JJ, Taupin D, Koh TJ, Chen D, Zhao CM, Podolsky DK & Wang TC (2002). TFF2/SP-deficient mice show decreased gastric proliferation, increased acid secretion, and increased susceptibility to NSAID injury. *J Clin Invest* **109,** 193-204.

Felding-Habermann B & Cheresh DA (1993). Vitronectin and its receptors. *Curr Opin Cell Biol* **6,** 864-868.

Feng J, Petersen CD, Coy DH, Jiang JK, Thomas CJ, Pollak MR & Wank SA (2010). Calcium-sensing receptor is a physiologic multimodal chemosensor regulating gastric G-cell growth and gastrin secretion. *Proc Natl Acad Sci U S A* **107,** 17791-17796.

Ferrara N (2004). Vascular endothelial growth factor: basic science and clinical progress. *Endocr Rev* **25,** 581-611.

Filaretova L, Bagaeva T & Morozova O (2012). Stress and the Stomach: Corticotropin-Releasing Factor May Protect the Gastric Mucosa in Stress Through Involvement of Glucocorticoids. *Cell Mol Neurobiol* **32,** 829-836.

Fiorucci S, Distrutti E, Cirino G & Wallace JL (2006). The emerging roles of hydrogen sulfide in the gastrointestinal tract and liver. *Gastroenterology* **131,** 259-271.

Fox JG, Li X, Cahill RJ, Andrutis K, Rustgi AK, Odze R & Wang T (1996). Hypertrophic gastropathy in *Helicobacter felis*–infected wild-type C57BL/6 mice and p53 hemizygous transgenic mice. *Gastoenterology* **110,** 155-166.

Fox JG, Dangler CA, Whary MT, Edelman W, Kucherlapati R & Wang TC (1997). Mice carrying a truncated Apc gene have diminished gastric epithelial proliferation, gastric inflammation, and humoral immunity in response to Helicobacter felis infection. *Cancer Res* **57,** 3972-3978.

Fox JG, Beck P, Dangler CA, Whary MT, Wang TC, Shi HN & Nagler-Anderson C (2000). Concurrent enteric helminth infection modulates inflammation and gastric immune responses and reduces helicobacter-induced gastric atrophy. *Nat Med* **6,** 536-542.

Fox JG, Sheppard BJ, Dangler CA, Whary MT, Ihrig M & Wang TC (2002). Germ-line p53-targeted disruption inhibits helicobacter-induced premalignant lesions and invasive gastric carcinoma through down-regulation of Th1 proinflammatory responses. *Cancer Res* **62,** 696-702.

Fox JG, Rogers AB, Ihrig M, Taylor NS, Whary MT, Dockray G, Varro A & Wang TC (2003). Helicobacter pylori-associated gastric cancer in INS-GAS mice is gender specific. *Cancer Res* **63,** 942-950.

Frenck RW Jr & Clemens J (2003). Helicobacter in the developing world. *Microbes Infect* **5,** 705-713.

Fu S, Ramanujam KS, Wong A, Fantry GT, Drachenberg CB, James SP, Meltzer SJ & Wilson KT (1999). Increased expression and cellular localization of inducible nitric oxide synthase and cyclooxygenase 2 in Helicobacter pylori gastritis. *Gastroenterology* **116,** 1319-1329.

Fuccio L, Zagari RM, Minardi ME & Bazzoli F (2007). Systematic review: Helicobacter pylori eradication for the prevention of gastric cancer. *Aliment Pharmacol Ther* **25,** 133-141.

Fujimiya T, Liu J, Kojima H, Shirafuji S, Kimura H & Fujimiya M (2009). Pathological roles of bone marrow-derived stellate cells in a mouse model of alcohol-induced fatty liver. *Am J Physiol Gastrointest Liver Physiol* **297,** G451-G460.

Fukaya M, Isohata N, Ohta H, Aoyagi K, Ochiya T, Saeki N, Yanagihara K, Nakanishi Y, Taniguchi H, Sakamoto H, Shimoda T, Nimura Y, Yoshida T & Sasaki H (2006). Hedgehog signal activation in gastric pit cell and in diffuse-type gastric cancer. *Gastroenterology* **131,** 14-29.

Fukuhara H, Kadowaki Y, Ose T, Monowar A, Imaoka H, Ishihara S, Takasawa S & Kinoshita Y (2010). In vivo evidence for the role of RegI in gastric regeneration: transgenic overexpression of RegI accelerates the healing of experimental gastric ulcers. *Lab Invest* **90,** 556-565.

Fukui H, Kinoshita Y, Maekawa T, Okada A, Waki S, Hassan S, Okamoto H & Chiba T (1998). Regenerating gene protein may mediate gastric mucosal proliferation induced by hypergastrinemia in rats. *Gastroenterology* **115,** 1483-1493.

Fukui T, Nishio A, Okazaki K, Uza N, Ueno S, Kido M, Inoue S, Kitamura H, Kiriya K, Ohashi S, Asada M, Tamaki H, Matsuura M, Kawasaki K, Suzuki K, Uchida K, Fukui H, Nakase H, Watanabe N & Chiba T (2006). Gastric mucosal hyperplasia via upregulation of gastrin induced by persistent activation of gastric innate immunity in major histocompatibility complex class II deficient mice. *Gut* **55,** 607-615.

Fukui T, Kishimoto M, Nakajima A Yamashina M, Nakayama S, Kusuda T, Sakaguchi Y, Yoshida K, Uchida K, Nishio A, Matsuzaki K & Okazaki K (2011). The specific linker phosphorylation of Smad2/3 indicates epithelial stem cells in stomach; particularly increasing in mucosae of Helicobacter-associated gastritis. *J Gastroenterol* **46,** 456-468.

Fuyuhiro Y, Yashiro M, Noda S, Kashiwagi S, Matsuoka J, Doi Y, Kato Y, Muguruma K, Sawada T & Hirakawa K (2010). Myofibroblasts are associated with the progression of scirrhous gastric carcinoma. *Exp Ther Med* **1,** 547-551.

Fuyuhiro Y, Yashiro M, Noda S, Kashiwagi S, Matsuoka J, Doi Y, Kato Y, Hasegawa T, Sawada T & Hirakawa K (2011). Upregulation of cancer-associated myofibroblasts by TGF-β from scirrhous gastric carcinoma cells. *Br J Cancer* **105,** 996-1001.

Fuyuhiro Y, Yashiro M, Noda S, Matsuoka J, Hasegawa T, Kato Y, Sawada T & Hirakawa K (2012). Cancer-associated orthotopic myofibroblasts stimulates the motility of gastric carcinoma cells. *Cancer Sci* **103,** 797-805.

Gabbiani G, Ryan GB & Majno G (1971). Presence of modified fibroblasts in granulation tissue and their possible role in wound contraction. *Experientia* **27,** 549-550.

Gambero A, Marostica M, Becker TL & Pedrazzoli J Jr (2007). Effect of different cyclooxygenase inhibitors on gastric adaptive cytoprotection induced by 20% ethanol. *Dig Dis Sci* **52,** 425-433.

Ganesh S, Sier CF, Heerding MM, van Krieken JH, Griffioen G, Welvaart K, van de Velde CJ, Verheijen JH, Lamers CB & Verspaget HW (1996). Prognostic value of the plasminogen activation system in patients with gastric carcinoma. *Cancer* **77,** 1035-1043.

Ganguly K, Maity P, Reiter RJ & Swarnakar S (2005). Effect of melatonin on secreted and induced matrix metalloproteinase-9 and -2 activity during prevention of indomethacin-induced gastric ulcer. *J Pineal Res* **39,** 307-315.

Ganguly K, Sharma AV, Reiter RJ & Swarnakar S (2010). Melatonin promotes angiogenesis during protection and healing of indomethacin-induced gastric ulcer: role of matrix metaloproteinase-2. *J Pineal Res* **49,** 130-140.

Ghosh AK, Bradham WS, Gleaves LA, De Taeye B, Murphy SB, Covington JW & Vaughan DE (2010). Genetic deficiency of plasminogen activator inhibitor-1 promotes cardiac fibrosis in aged mice: involvement of constitutive transforming growth factor-beta signaling and endothelial-to-mesenchymal transition. *Circulation* **122,** 1200-1209.

Ghosh AK & Vaughan DE (2012). PAI-1 in tissue fibrosis. *J Cell Physiol* **227,** 493-507.

Giannakis M, Chen SL, Karam SM, Engstrand L & Gordon JI (2008). Helicobacter pylori evolution during progression from chronic atrophic gastritis to gastric cancer and its impact on gastric stem cells. *Proc Natl Acad Sci U S A* **105,** 4358-4363.

Giannella RA, Broitman SA & Zamcheck N (1972). Gastric acid barrier to ingested microorganisms in man: studies in vivo and in vitro. *Gut* **13,** 251-256.

Glocker EC, Lange N, Covacci A, Bereswill S, Kist M & Pahl HL (1998). Proteins encoded by the cag pathogenicity island of Helicobacter pylori are required for NF-κB activation. *Infect Immun* **66,** 2346-2348.

Goddard PJ, Kao YC & Lichtenberger LM (1990). Luminal surface hydrophobicity of canine gastric mucosa is dependent on a surface mucous gel. *Gastroenterology* **98,** 361-370.

Goldenring JR, Ray GS, Soroka CJ, Smith J, Modlin IM, Meise KS & Coffey RJ Jr (1996). Overexpression of transforming growth factor-alpha alters differentiation of gastric cell lineages. *Dig Dis Sci* **41,** 773-784.

Gong Y, Hart E, Shchurin A & Hoover-Plow J (2008). Inflammatory macrophage migration requires MMP-9 activation by plasminogen in mice. *J Clin Invest* **118,** 3012-3024.

Goolaerts A, Lafargue M, Song Y, Miyazawa B, Arjomandi M, Carlès M, Roux J, Howard M, Parks DA, Iles KE & Pittet JF (2011). PAI-1 is an essential component of the pulmonary host response during Pseudomonas aeruginosa pneumonia in mice. *Thorax* **66,** 788-796.

Guillemin K, Salama NR, Tompkins LS & Falkow S (2002). Cag pathogenicity island-specific responses of gastric epithelial cells to Helicobacter pylori infection. *Proc Natl Acad Sci U S A* **99,** 15136-15141.

Gutiérrez A, Sánchez-Payá J, Marco P & Pérez-Mateo M (2001). Prognostic value of fibrinolytic tests for hospital outcome in patients with acute upper gastrointestinal hemorrhage. *J Clin Gastroenterol* **32,** 315-318.

Gutierrez LS, Schulman A, Brito-Robinson T, Noria F, Ploplis VA & Castellino FJ (2000). Tumor development is retarded in mice lacking the gene for urokinase-type plasminogen activator or its inhibitor, plasminogen activator inhibitor-1. *Cancer Res* **60,** 5839-5847.

Gutierrez-Gonzalez L, Graham TA, Rodriguez-Justo M, Leedham SJ, Novelli MR, Gay LJ, Ventayol-Garcia T, Green A, Mitchell I, Stoker DL, Preston SL, Bamba S, Yamada E, Kishi Y, Harrison R, Jankowski JA, Wright NA & McDonald SA (2011). The clonal origins of dysplasia from intestinal metaplasia in the human stomach. *Gastroenterology* **140,** 1251-1260.

Guth PH (1992). Current concepts in gastric microcirculatory pathophysiology. *Yale J Biol Med* **65,** 677-688.

Gyetko MR, Sitrin RG, Fuller JA, Todd RF 3rd, Petty H & Standiford TJ (1995) Function of the urokinase receptor (CD87) in neutrophil chemotaxis. *J Leukoc Biol* **58,** 533-538.

Gyetko MR, Chen GH, McDonald RA, Goodman R, Huffnagle GB, Wilkinson CC, Fuller JA & Toews GB (1996). Urokinase is required for the pulmonary inflammatory response to Cryptococcus neoformans. A murine transgenic model. *J Clin Invest* **97,** 1818-1826.

Gyetko MR, Sud S, Kendall T, Fuller JA, Newstead MW & Standiford TJ (2000). Urokinase receptor-deficient mice have impaired neutrophil recruitment in response to pulmonary Pseudomonas aeruginosa infection. *J Immunol* **165,** 1513-1519.

Gyetko MR, Sud S, Sonstein J, Polak T, Sud A & Curtis JL (2001). Antigen-driven lymphocyte recruitment to the lung is diminished in the absence of urokinase-type plasminogen activator (uPA) receptor, but is independent of uPA. *J Immunol* **167,** 5539-5542.

Gyetko MR, Sud S, Chen GH, Fuller JA, Chensue SW & Toews GB (2002). Urokinase-type plasminogen activator is required for the generation of a type 1 immune response to pulmonary Cryptococcus neoformans infection. *J Immunol* **168,** 801-809.

Gyetko MR, Sud S & Chensue SW (2004). Urokinase-deficient mice fail to generate a type 2 immune response following schistosomal antigen challenge. *Infect Immun* **72,** 461-467.

Haid D, Widmayer P & Breer H (2011). Nutrient sensing receptors in gastric endocrine cells. *J Mol Histol* **42,** 355-364.

Hansen OH, Pedersen T, Larsen JK & Rehfeld JF (1976). Effect of gastrin on gastric mucosal cell proliferation in man. *Gut* **17,** 536-541.

Harbeck N, Kates RE, Gauger K, Willems A, Kiechle M, Magdolen V & Schmitt M (2004). Urokinase-type plasminogen activator (uPA) and its inhibitor PAI-I: novel tumor-derived factors with a high prognostic and predictive impact in breast cancer. *Thromb Haemost* **91,** 450-456.

Hattori N, Mizuno S, Yoshida Y, Chin K, Mishima M, Sisson TH, Simon RH, Nakamura T & Miyake M (2004). The plasminogen activation system reduces fibrosis in the lung by a hepatocyte growth factor-dependent mechanism. *Am J Pathol* **164,** 1091-1098.

Hawkey CJ, Kemp RT, Walt RP, Bhaskar NK, Davies J & Filipowicz B (1988). Evidence that adaptive cytoprotection in rats is not mediated by prostaglandins. *Gastroenterology* **94,** 948-954.

Hayashi S & McMahon AP (2002). Efficient recombination in diverse tissues by a tamoxifen-inducible form of Cre: a tool for temporally regulated gene activation/inactivation in the mouse. *Dev Biol* **244,** 305-318.

Hayashi Y, Tsuji S, Tsujii M, Nishida T, Ishii S, Iijima H, Nakamura T, Eguchi H, Miyoshi E, Hayashi N & Kawano S (2008). Topical transplantation of mesenchymal stem cells accelerates gastric ulcer healing in rats. *Am J Physiol Gastrointest Liver Physiol* **294,** G778-G786.

He H, Yim M, Liu KH, Cody SC, Shulkes A & Baldwin GS (2008). Involvement of G proteins of the Rho family in the regulation of Bcl-2-like protein expression and caspase 3 activation by Gastrins. *Cell Signal* **20,** 83-93.

He XC, Zhang J, Tong WG, Tawfik O, Ross J, Scoville DH, Tian Q, Zeng X, He X, Wiedemann LM, Mishina Y & Li L (2004). BMP signaling inhibits intestinal stem cell self-renewal through suppression of Wnt-beta-catenin signaling. *Nat Genet* **36,** 1117-1121.

He Y, Liu XD, Chen ZY, Zhu J, Xiong Y, Li K, Dong JH & Li X (2007). Interaction between cancer cells and stromal fibroblasts is required for activation of the uPAR-uPA-MMP-2 cascade in pancreatic cancer metastasis. *Clin Cancer Res* **13,** 3115-3124.

Heinemann A, Jocic M, Peskar BM & Holzer P (1996). CCK-evoked hyperemia in rat gastric mucosa in involves neural mechanisms and nitric oxide. *Am J Physiol Gastrointest Liver Physiol* **270,** G253-G258.

Heiss MM, Babic R, Allgayer H, Greutzner KU, Jauch K-W, Loehrs U & Schildberg FW (1995). Tumor-associated proteolysis and prognosis: new functional risk factors in gastric cancer defined by the urokinase-type plasminogen activator system. *J Clin Oncol* **13,** 2084-2093.

Hemers E, Duval C, McCaig C, Handley M, Dockray GJ & Varro A (2005). Insulin-like growth factor binding protein-5 is a target of matrix metalloproteinase-7: implications for epithelial-mesenchymal signaling. *Cancer Res* **65,** 7363-7369.

Henagan JM, Smith GS, Seidel ER & Miller TA (1984). Influence of vagotomy on mucosal protection against alcohol-induced gastric damage in the rat. *Gastroenterology* **87,** 903-908.

Hernández-Díaz S & Rodríguez LA (2000). Association between nonsteroidal anti-inflammatory drugs and upper gastrointestinal tract bleeding/perforation: an overview of epidemiologic studies published in the 1990s. *Arch Intern Med* **160,** 2093-2099.

Herszényi L, Plebani M, Cardin R, Carraro P, De Paoli M, Roveroni G, Naccarato R & Farinati F (1995). The role of urokinase-type plasminogen activator and its inhibitor PAI-1 in gastric cancer. *Acta Physiol Hung* **83,** 213-221.

Herszenyi L, Plebani M, Carraro P, De Paoli M, Cardin R, Di Mario F, Kusstatscher S, Naccarato R & Farinati F (1997). Impaired fibrinolysis and increased protease levels in gastric and duodenal mucosa of patients with active duodenal ulcer. *Am J Gastroenterol* **92,** 843-847.

Heymans S, Pauschinger M, De Palma A, Kallwellis-Opara A, Rutschow S, Swinnen M, Vanhoutte D, Gao F, Torpai R, Baker AH, Padalko E, Neyts J, Schultheiss HP, Van de Werf F, Carmeliet P & Pinto YM (2006). Inhibition of urokinase-type plasminogen activator or matrix metalloproteinases prevents cardiac injury and dysfunction during viral myocarditis. *Circulation* **114,** 565-573.

Higashi H, Nakaya A, Tsutsumi R, Yokoyama K, Fujii Y, Ishikawa S, Higuchi M, Takahashi A, Kurashima Y, Teishikata Y, Tanaka S, Azuma T & Hatakeyama M (2004). Helicobacter pylori CagA induces Ras-independent morphogenetic response through SHP-2 recruitment and activation. *J Biol Chem* **279,** 17205-17216.

Higgs GA, Salmon JA, Henderson B & Vane JR (1987). Pharmacokinetics of aspirin and salicylate in relation to inhibition of arachidonate cyclooxygenase and antiinflammatory activity. *Proc Natl Acad Sci U S A* **84,** 1417-1420.

Higham AD, Bishop LA, Dimaline R, Blackmore CG, Dobbins AC, Varro A, Thompson DG & Dockray GJ (1999). Mutations of RegIalpha are associated with enterochromaffin-like cell tumor development in patients with hypergastrinemia. *Gastroenterology* **116,** 1310-1318.

Higham J, Kang JY & Majeed A (2002). Recent trends in admissions and mortality due to peptic ulcer in England: increasing frequency of haemorrhage among older subjects. *Gut* **50,** 460-464.

Hildenbrand R, Gandhari M, Stroebel P, Marx A, Allgayer H & Arens N (2008). The urokinase-system — role of cell proliferation and apoptosis. *Histol Histopathol* **23,** 227-236.

Hinz B (2010). The myofibroblast: paradigm for a mechanically active cell. *J Biomech* **43,** 146-155.

Hinz B, Phan SH, Thannickal VJ, Prunotto M, Desmoulière A, Varga J, De Wever O, Mareel M & Gabbiani G (2012). Recent developments in myofibroblast biology: paradigms for connective tissue remodeling. *Am J Pathol* **180,** 1340-1355.

Hirata I, Naito Y, Handa O, Hayashi N, Mizushima K, Adachi S, Omatsu T, Okayama T, Kishimoto E, Ichikawa H, Takagi T, Kokura S, Otaka M & Yoshikawa T (2009). Heat-shock protein 70-overexpressing gastric epithelial cells are resistant to indomethacin-induced apoptosis. *Digestion* **79,** 243-250.

Hoffmann W (2005). Trefoil factors TFF (trefoil factor family) peptide-triggered signals promoting mucosal restitution. *Cell Mol Life Sci* **62,** 2932-2938.

Holmberg C, Quante M, Steele I, Kumar JD, Balabanova S, Duval C, Czepan M, Rakonczay Z Jr, Tiszlavicz L, Nemeth I, Lazar G, Simonka Z, Jenkins R, Hegyi P, Wang TC, Dockray GJ & Varro A (2012). Release of TGFβig-h3 by gastric myofibroblasts slows tumor growth and is decreased with cancer progression. *Carcinogenesis* **33,** 1553-1562.

Holmberg C, Ghesquière B, Impens F, Gevaert K, Kumar JD, Cash N, Kandola S, Hegyi P, Wang TC, Dockray GJ & Varro A (2013). Mapping proteolytic processing in the secretome of gastric cancer-associated myofibroblasts reveals activation of MMP-1, MMP-2, and MMP-3. *J Proteome Res* **12,** 3413-3422.

Hooker DJ, Mobarok M, Anderson JL, Rajasuriar R, Gray LR, Ellett AM, Lewin SR, Gorry PR & Cherry CL (2012). A new way of measuring apoptosis by absolute quantitation of inter-nucleosomally fragmented genomic DNA. *Nucleic Acids Res* **40,** e113.

Horn A, Palumbo K, Cordazzo C, Dees C, Akhmetshina A, Tomcik M, Zerr P, Avouac J, Gusinde J, Zwerina J, Roudaut H, Traiffort E, Ruat M, Distler O, Schett G & Distler JH (2012). Hedgehog signaling controls fibroblast activation and tissue fibrosis in systemic sclerosis. *Arthritis Rheum* **64,** 2724-2733.

Horowitz JC, Rogers DS, Simon RH, Sisson TH & Thannickal VJ (2008). Plasminogen activation induced pericellular fibronectin proteolysis promotes fibroblast apoptosis. *Am J Respir Cell Mol Biol* **38,** 78-87.

Houck KA, Leung DW, Rowland AM, Winer J & Ferrara N (1992). Dual regulation of vascular endothelial growth factor bioavailability by genetic and proteolytic mechanisms. *J Biol Chem* **267,** 26031-26037.

Houghton J, Stoicov C, Nomura S, Rogers AB, Carlson J, Li H, Cai X, Fox JG, Goldenring JR & Wang TC (2004). Gastric cancer originating from bone marrow-derived cells. *Science* **306,** 1568-1571.

Hu PF, Chen H, Zhong W, Lin Y, Zhang X, Chen YX & Xie WF (2009). Adenovirus-mediated transfer of siRNA against PAI-1 mRNA ameliorates hepatic fibrosis in rats. *J Hepatol* **51,** 102-113.

Huai Q, Mazar AP, Kuo A, Parry GC, Shaw DE, Callahan J, Li Y, Yuan C, Bian C, Chen L, Furie B, Furie BC, Cines DB & Huang M (2006). Structure of human plasminogen activator in complex with its receptor. *Science* **311,** 656-669.

Huai Q, Zhou A, Lin L, Mazar AP, Parry GC, Callahan J, Shaw DE, Furie B, Furie BC & Huang M (2008). Crystal structures of two human vitronectin, urokinase and urokinase receptor complexes. *Nat Struct Mol Biol* **15,** 422-423.

Hubbell E, Liu WM & Mei R (2002). Robust estimators for expression analysis. *Bioinformatics* **18,** 1585-1592.

Huh WJ, Khurana SS, Geahlen JH, Kohli K, Waller RA & Mills JC (2012). Tamoxifen induces rapid, reversible atrophy, and metaplasia in mouse stomach. *Gastroenterology* **142,** 21-24.

Ikeda A, Gabazza EC, Morser J, Imoto I, Kuroda M, D'Alessandro-Gabazza CN, Hara K, Ruiz DB, Bernabe PG, Katsurahara M, Toda M, Kobayashi Y, Yano Y, Sumida Y, Suzuki K, Taguchi O & Takei Y (2009). Presence of thrombin-activatable fibrinolysis inhibitor in Helicobacter pylori-associated gastroduodenal disease. *Helicobacter* **14,** 147-155.

Illemann M, Bird N, Majeed A, Laerum OD, Lund LR, Danø K & Nielsen BS (2009). Two distinct expression patterns of urokinase, urokinase receptor and plasminogen activator inhibitor-1 in colon cancer liver metastases. *Int J Cancer* **124,** 1860-1870.

Irish JM, Hovland R, Krutzik PO, Perez OD, Bruserud Ø, Gjertsen BT & Nolan GP (2004). Single cell profiling of potentiated phospho-protein networks in cancer cells. *Cell* **118,** 217-228.

Ito T, Kobayashi D, Uchida K, Takemura T, Nagaoka S, Kobayashi I, Yokoyama T, Ishige I, Ishige Y, Ishida N, Furukawa A, Muraoka H, Ikeda S, Sekine M, Ando N, Suzuki Y, Yamada T, Suzuki T & Eishi Y (2008). Helicobacter pylori invades the gastric mucosa and translocates to the gastric lymph nodes. *Lab Invest* **88,** 664-681.

Iwamoto J, Takahashi K, Mizokami Y, Otsubo T, Miura S, Narasaka T, Takeyama H, Omata T, Shimokoube K & Matsuoka T (2003). Expression of urokinase-type plasminogen activator and its receptor in gastric fibroblasts and effects of nonsteroidal antiinflammatory drugs and prostaglandin. *Dig Dis Sci* **48,** 2247-2256.

Iwamoto J, Mizokami Y, Takahashi K, Nakajima K, Ohtsubo T, Miura S, Narasaka T, Takeyama H, Omata T, Shimokobe K, Ito M, Takehara H & Matsuoka T (2005). Expressions of urokinase-type plasminogen activator, its receptor and plasminogen activator inhibitor-1 in gastric cancer cells and effects of Helicobacter pylori. *Scand J Gastroenterol* **40,** 783-793.

Iwamoto J, Mizokami Y, Takahashi K, Matsuoka T & Matsuzaki Y (2008). The effects of cyclooxygenase2-prostaglandinE2 pathway on Helicobacter pylori-induced urokinase-type plasminogen activator system in the gastric cancer cells. *Helicobacter* **13,** 174-182.

Jain RN & Samuelson LC (2006). Differentiation of the gastric mucosa. II. Role of gastrin in gastric epithelial cell proliferation and maturation. *Am J Physiol Gastrointest Liver Physiol* **291,** G762-G765.

Jenks PJ, Jeremy AH, Robinson PA, Walker MM & Crabtree JE (2003). Long-term infection with Helicobacter felis and inactivation of the tumour suppressor gene p53 cumulatively enhance the gastric mutation frequency in Big Blue transgenic mice. *J Pathol* **201,** 596-602.

Jespersen J & Astrup T (1983). A study of the fibrin plate assay of fibrinolytic agents. Optimal conditions, reproducibility and precision. *Haemostasis* **13,** 301-315.

Jing Y, Kovacs K, Kurisetty V, Jiang Z, Tsinoremas N & Merchan JR (2012). Role of plasminogen activator inhibitor-1 in urokinase's paradoxical in vivo tumor suppressing or promoting effects. *Mol Cancer Res* **10,** 1271-1281.

Jo M, Takimoto S, Montel V & Gonias SL (2009). The urokinase receptor promotes cancer metastasis independently of urokinase-type plasminogen activator in mice. *Am J Pathol* **175,** 190-200.

Jo M, Eastman BM, Webb DL, Stoletov K, Klemke R & (2010). Cell signaling by urokinase-type plasminogen activator receptor induces stem cell-like properties in breast cancer cells. *Cancer Res* **70,** 8948-8958.

Johnson LR (1977). New aspects of the trophic action of gastrointestinal hormones. *Gastroenterology* **72,** 788-792.

Jones MK, Padilla OR, Webb NA & Norng M (2008). The anti-apoptosis protein, survivin, mediates gastric epithelial cell cytoprotection against ethanol-induced injury via activation of the p34 (cdc2) cyclin-dependent kinase. *J Cell Physiol* **215,** 750-764.

Kaneko T, Konno H, Baba M, Tanaka T & Nakamura S (2003). Urokinase-type plasminogen activator expression correlates with tumor angiogenesis and poor outcome in gastric cancer. *Cancer Sci* **94,** 43-49.

Kang DH, Han ME, Song MH, Lee YS, Kim EH, Kim HJ, Kim GH, Kim DH, Yoon S, Baek SY, Kim BS, Kim JB & Oh SO (2009). The role of hedgehog signaling during gastric regeneration. *J Gastroenterol* **44,** 372-379.

Kanno Y, Kaneiwa A, Minamida M, Kanno M, Tomogane K, Takeuchi K, Okada K, Ueshima S, Matsuo O & Matsuno H. (2008). The absence of uPAR is associated with the progression of dermal fibrosis. *J Invest Dermatol* **128,** 2792-2797.

Karadi O, Bodis B, Szabo I, Rumi G & Mozsik G (1999). Difference between the effect of acute and chronic surgical vagotomy on the cytoprotective action of atropine against indomethacin-induced mucosal lesions on the gastrointestinal tract in rats. *J Physiol Paris* **93,** 487-490.

Karadi O, Nagy Z, Bodis B & Mozsik G (2001). Atropine-induced gastrointestinal cytoprotection dependences to the intact of vagal nerve against indomethacin-induced gastrointestinal mucosal and microvascular damage in rats. *J Physiol Paris* **95,** 29-33.

Karam SM & Leblond CP (1992). Identifying and counting epithelial cell types in the “corpus” of the mouse stomach. *Anat Rec* **232,** 231-246.

Karam SM (1993). Dynamics of epithelial cells in the corpus of the mouse stomach. IV. Bidirectional migration of parietal cells ending in their gradual degeneration and loss. *Anat Rec* **236,** 314-332.

Karam SM & Leblond CP (1993a). Dynamics of epithelial cells in the corpus of the mouse stomach. V. Behavior of enteroendocrine and caveolated cells: general conclusions on cell kinetics in the oxyntic epithelium. *Anat Rec* **236,** 333-340.

Karam SM & Leblond CP (1993b). Dynamics of epithelial cells in the corpus of the mouse stomach. I. Identification of proliferative cell types and pinpointing of the stem cell. *Anat Rec* **236,** 259-279.

Karam SM & Leblond CP (1993c). Dynamics of epithelial cells in the corpus of the mouse stomach. II. Outward migration of pit cells. *Anat Rec* **236,** 280-296.

Karam SM & Leblond CP (1993d). Dynamics of epithelial cells in the corpus of the mouse stomach. III. Inward migration of neck cells followed by progressive transformation into zymogenic cells. *Anat Rec* **236,** 297-313.

Karam SM, Straiton T, Hassan WM & Leblond CP (2003). Defining epithelial cell progenitors in the human oxyntic mucosa. *Stem Cells* **21,** 322-336.

Kargman S, Charleson S, Cartwright M, Frank J, Riendeau D, Mancini J, Evans J & O'Neill G (1996). Characterization of Prostaglandin G/H Synthase 1 and 2 in rat, dog, monkey, and human gastrointestinal tracts. *Gastroenterology* **111,** 445-454.

Katano T, Ootani A, Mizoshita T, Tanida S, Tsukamoto H, Ozeki K, Ebi M, Mori Y, Kataoka H, Kamiya T, Toda S & Joh T (2013). Establishment of a long-term three-dimensional primary culture of mouse glandular stomach epithelial cells within the stem cell niche. *Biochem Biophys Res Commun* **432,** 558-563.

Kato T, Nagatsu T, Kimura T & Sakakibara S (1978). Fluorescence assay of x-prolyl dipeptidyl-aminopeptidase activity with a new fluorogenic substrate. *Biochem Med* **19,** 351-359.

Kawakubo K, Yang H & Taché Y (2002). Gastric protective effect of peripheral PYY through PYY preferring receptors in anesthetized rats. *Am J Physiol Gastrointest Liver Physiol* **283,** G1035-G1041.

Kawasaki K, Hayashi Y, Wang Y, Suzuki S, Morita Y, Nakamura T, Narita K, Doe W, Itoh H & Kuroda Y (1998). Expression of urokinase-type plasminogen activator receptor and plasminogen activator inhibitor-1 in gastric cancer. *J Gastroenterol Hepatol* **13,** 936-944.

Kazumori H, Ishihara S, Kawashima K, Fukuda R, Chiba T & Kinoshita Y (2001). Analysis of gastrin receptor gene expression in proliferating cells in the neck zone of gastric fundic glands using laser capture microdissection. *FEBS Lett* **489,** 208-214.

Keates AC, Tummala S, Peek RM Jr, Csizmadia E, Kunzli B, Becker K, Correa P, Romero-Gallo J, Piazuelo MB, Sheth S, Kelly CP, Robson SC & Keates S (2008). Helicobacter pylori infection stimulates plasminogen activator inhibitor 1 production by gastric epithelial cells. *Infect Immun* **76,** 3992-3999.

Kenny S (2008). The regulation of plasminogen activator inhibitor-l (PAI-l) and other uPA system members in the gastric epithelium by Helicobacter pylori. *PhD thesis, University of Liverpool, UK.*

Kenny S, Duval C, Sammut SJ, Steele I, Pritchard DM, Atherton JC, Argent RH, Dimaline R, Dockray GJ & Varro A (2008). Increased expression of the urokinase plasminogen activator system by Helicobacter pylori in gastric epithelial cells. *Am J Physiol Gastrointest Liver Physiol* **295,** G431-G441.

Kenny S, Gamble J, Lyons S, Vlotkovic N, Dimaline R, Varro A & Dockray GJ (2013a). Gastric expression of plasminogen activator inhibitor (PAI)-1 in mice is associated with hyperphagia and obesity. *Endocrinology* **154,** 718-726.

Kenny S, Steele I, Lyons S, Moore AR, Murugesan SV, Tiszlavicz L, Dimaline R, Pritchard DM, Varro A & Dockray GJ (2013b). The role of plasminogen activator inhibitor-1 in gastric mucosal protection. *Am J Physiol Gastrointest Liver Physiol* **304,** G814-G822.

Khurana SS, Riehl TE, Moore BD, Fassan M, Rugge M, Romero-Gallo J, Noto J, Peek RM Jr, Stenson WF & Mills JC (2013). The hyaluronic acid receptor CD44 coordinates normal and metaplastic gastric epithelial progenitor cell proliferation. *J Biol Chem* **288,** 16085-16097.

Kidd M, Tang LH, Modlin IM, Zhang T, Chin K, Holt PR & Moss SF (2000). Gastrin-mediated alterations in gastric epithelial apoptosis and proliferation in a mastomys rodent model of gastric neoplasia. *Digestion* **62,** 143-151.

Kim DH, Kim SW, Song YJ, Oh TY, Han SU, Kim YB, Joo HJ, Cho YK, Kim DY, Cho SW, Kim MW, Kim JH & Hahm KB (2003). Long-term evaluation of mice model infected with Helicobacter pylori: focus on gastric pathology including gastric cancer. *Aliment Pharmacol Ther* **18 Suppl 1,** 14-23.

Kim JS, Chang JH, Chung SI & Yum JS (2001). Importance of the host genetic background on immune responses to Helicobacter pylori infection and therapeutic vaccine efficacy. *FEMS Immunol Med Microbiol* **31,** 41-46.

Kim KM, Oh YL, Ko JS, Choe YH & Seo JK (2004a). Histopathology and expression of Ki-67 and cyclooxygenase-2 in childhood Helicobacter pylori gastritis. *J Gastroenterol* **39,** 231-237.

Kim MH, Yoo HS, Chang HJ, Hong MH, Kim HD, Chung IJ, Shin BA, Cho MJ, Ahn BW & Jung YD (2005). Urokinase plasminogen activator receptor is upregulated by Helicobacter pylori in human gastric cancer AGS cells via ERK, JNK, and AP-1. *Biochem Biophys Res Commun* **333,** 874-880.

Kim MH, Yoo HS, Kim MY, Jang HJ, Baek MK, Kim HR, Kim KK, Shin BA, Ahn BW & Jung YD (2007). Helicobacter pylori stimulates urokinase plasminogen activator receptor expression and cell invasiveness through reactive oxygen species and NF-kappaB signaling in human gastric carcinoma cells. *Int J Mol Med* **19,** 689-697.

Kim MH, Park JS, Chang HJ, Baek MK, Kim HR, Shin BA, Ahn BW & Jung YD (2008). Lysophosphatidic acid promotes cell invasion by up-regulating the urokinase-type plasminogen activator receptor in human gastric cancer cells. *J Cell Biochem* **104,** 1102-1112.

Kim SJ, Park YS, Paik HD & Chang HI (2011). Effect of anthocyanins on expression of matrix metalloproteinase-2 in naproxen-induced gastric ulcers. *Br J Nutr* **106,** 1792-1801.

Kim TH & Shivdasani RA (2011). Notch signaling in stomach epithelial stem cell homeostasis. *J Exp Med* **208,** 677-688.

Kim TY, Jong HS, Jung Y, Kim TY, Kang GH & Bang YJ (2004b). DNA hypermethylation in gastric cancer. *Aliment Pharmacol Ther* **20 Suppl 1,** 131-142.

Kirton CM, Wang T & Dockray GJ (2002). Regulation of parietal cell migration by gastrin in the mouse. *Am J Physiol Gastrointest Liver Physiol* **283,** G787-G793.

Kita Y, Fukagawa T, Mimori K, Kosaka Y, Ishikawa K, Aikou T, Natsugoe S, Sasako M & Mori M (2009). Expression of uPAR mRNA in peripheral blood is a favourite marker for metastasis in gastric cancer cases. *Br J Cancer* **100,** 153-159.

Kitadai Y, Sasaki A, Ito M, Tanaka S, Oue N, Yasui W, Aihara M, Imagawa K, Haruma K & Chayama K (2003). Helicobacter pylori infection influences expression of genes related to angiogenesis and invasion in human gastric carcinoma cells. *Biochem Biophys Res Commun* **311,** 809-814.

Kjoller L & Hall A (2001). Rac Mediates Cytoskeletal Rearrangements and Increased Cell Motility Induced by Urokinase-type Plasminogen Activator Receptor Binding to Vitronectin. *J Cell Biol* **152,** 1145-1157.

Kjoller L (2002). The urokinase plasminogen activator receptor in the regulation of the actin cytoskeleton and cell motility. *Biol Chem* **383,** 5-19.

Knapp TR, Daniels RJ & Kaplan EN (1977) Pathologic scar formation. Morphologic and biochemical correlates. *Am J Pathol* **86,** 47-69.

Konturek PC (1997). Physiological, immunohistochemical and molecular aspects of gastric adaptation to stress, aspirin and to H. pylori-derived gastrotoxins. *J Physiol Pharmacol* **48,** 3-42.

Konturek PC, Kania J, Kukharsky V, Ocker S, Hahn EG & Konturek SJ (2003). Influence of gastrin on the expression of cyclooxygenase-2, hepatocyte growth factor and apoptosis-related proteins in gastric epithelial cells. *J Physiol Pharmacol* **54,** 17-32.

Konturek PC, Brzozowski T, Pajdo R, Nikiforuk A, Kwiecien S, Harsch I, Drozdowicz D, Hahn EG & Konturek SJ (2004). Ghrelin-a new gastroprotective factor in gastric mucosa. *J Physiol Pharmacol* **55,** 325-336.

Konturek PC, Konturek SJ, Celinski K, Slomka M, Cichoz-Lach H, Bielanski W & Reiter RJ (2010). Role of melatonin in mucosal gastroprotection against aspirin-induced gastric lesions in humans. *J Pineal Res* **48,** 318-323.

Konturek SJ, Brzozowski T, Piastucki I, Radecki T, Dembinski A & Dembinska-Kiec A (1982). Role of locally generated prostaglandins in adaptive gastric cytoprotection. *Dig Dis Sci* **27,** 967-971.

Konturek SJ (1990). Role of growth factors in gastroduodenal protection and healing of peptic ulcers. *Gastroenterol Clin North Am* **19,** 41-65.

Konturek SJ, Brzozowski T, Pytko-Polonczyk J & Drozdowicz D (1995). Exogenous and endogenous cholecystokinin protects gastric mucosa against the damage caused by ethanol in rats. *Eur J Pharmacol* **273,** 57-62.

Kortlever RM, Higgins PJ & Bernards R (2006). Plasminogen activator inhibitor-1 is a critical downstream target of p53 in the induction of replicative senescence. *Nat Cell Biol* **8,** 877-888.

Koshelnick Y, Ehart M, Hufnagl P, Heinrich PC & Binder BR (1997). Urokinase receptor is associated with the components of the JAK1/STAT1 signaling pathway and leads to activation of this pathway upon receptor clustering in the human kidney epithelial tumor cell line TCL-598. *J Biol Chem* **272,** 28563-28567.

Kowal K, Bodzenta-Lukaszyk A, Pampuch A, Szmitkowski M, Donati MB & Iacoviello L (2007). Plasminogen activator inhibitor-1 plasma concentration in allergic asthma patients during allergen challenge. *Int Arch Allergy Immunol* **144,** 240-246.

Kowal K, Moniuszko M, Zukowski S & Bodzenta-Lukaszyk A (2010). Concentrations of plasminogen activator inhibitor-1 (PAI-1) and urokinase plasminogen activator (uPA) in induced sputum of asthma patients after allergen challenge. *Folia Histochem Cytobiol* **48,** 518-23.

Kuhli C, Luchtenberg M, Scharrer I & Hattenbach LO (2005). Massive subhyaloidal hemorrhage associated with severe PAI-1 deficiency. *Graefes Arch Clin Exp Ophthalmol* **243,** 963-966.

Kuipers EJ, Pérez-Pérez GI, Meuwissen SG & Blaser MJ (1995). Helicobacter pylori and atrophic gastritis: importance of the cagA status. *J Natl Cancer Inst* **87,** 1777-1780.

Kurose I, Miura S, Suematsu M, Asako H, Serizawa H, Ogata H, Hibi T & Tsuchiya M (1990). Possible involvement of tissue-type plasminogen activator in gastric ulcer formation - biochemical evaluation using biopsy specimens from gastric mucosa. *Dig Endosc* **2,** 49-53.

Kurose I, Miura S, Fukumura D, Suzuki M, Nagata H, Sekizuka E, Morishita T & Tsuchiya M (1994). Attenuating effect of antithrombin III on the fibrinolytic activation and microvascular derangement in rat gastric mucosa. *Thromb Haemost* **71,** 119-123.

Kwaan HC, Wang J, Svoboda K & Declerck PJ (2000). Plasminogen activator inhibitor 1 may promote tumour growth through inhibition of apoptosis. *Br J Cancer* **82,** 1702-1708.

Laaroubi K, Delbé J, Vacherot F, Desgranges P, Tardieu M, Jaye M, Barritault D & Courty J (1994). Mitogenic and in vitro angiogenic activity of human recombinant heparin affin regulatory peptide. *Growth Factors* **10,** 89-98.

Lademann U, Rømer MU, Jensen PB, Hofland KF, Larsen L, Christensen IJ & Brünner N (2005). Malignant transformation of wild-type but not plasminogen activator inhibitor-1 gene-deficient fibroblasts decreases cellular sensitivity to chemotherapy-mediated apoptosis. *Eur J Cancer* **41,** 1095-1100.

Laine L, Takeuchi K & Tarnawski A (2008). Gastric mucosal defense and cytoprotection: bench to bedside. *Gastroenterology* **135,** 41-60.

Lanas A, Perez-Aisa MA, Feu F, Ponce J, Saperas E, Santolaria S, Rodrigo L, Balanzo J, Bajador E, Almela P, Navarro JM, Carballo F, Castro M, Quintero E; Investigators of the Asociación Española de Gastroenterología (AEG) (2005). A nationwide study of mortality associated with hospital admission due to severe gastrointestinal events and those associated with nonsteroidal antiinflammatory drug use. *Am J Gastroenterol* **100,** 1685-1693.

Lawrence DA, Olson ST, Palaniappan S & Ginsburg D (1994). Engineering plasminogen activator inhibitor 1 mutants with increased functional stability *Biochemistry* **33,** 3643-3648.

Lee A, O'Rourke J, De Ungria MC, Robertson B, Daskalopoulos G & Dixon MF (1997). A standardized mouse model of *Helicobacter pylori* infection: introducing the Sydney strain. *Gastroenterology* **112,** 1386-1397.

Lee DH, Yang Y, Lee SJ, Kim KY, Koo TH, Shin SM, Song KS, Lee YH, Kim YJ, Lee JJ, Choi I & Lee JH (2003). Macrophage inhibitory cytokine-1 induces the invasiveness of gastric cancer cells by up-regulating the urokinase-type plasminogen activator system. *Cancer Res* **63,** 4648-4655.

Lee ER (1985a). Dynamic histology of the antral epithelium in the mouse stomach: I. Architecture of antral units. *Am J Anat* **172,** 187-204.

Lee ER (1985b). Dynamic histology of the antral epithelium in the mouse stomach: III. Ultrastructure and renewal of pit cells. *Am J Anat* **172,** 225-240.

Lee ER & Leblond CP (1985a). Dynamic histology of the antral epithelium in the mouse stomach: II. Ultrastructure and renewal of isthmal cells. *Am J Anat* **172,** 205-224.

Lee ER & Leblond CP (1985b). Dynamic histology of the antral epithelium in the mouse stomach: IV. Ultrastructure and renewal of gland cells. *Am J Anat* **172,** 241-259.

Lee MH, Vosburgh E, Anderson K & McDonagh J (1993). Deficiency of plasma plasminogen activator inhibitor 1 results in hyperfibrinolytic bleeding. *Blood* **81,** 2357-2362.

Leik CE, Su EJ, Nambi P, Crandall DL & Lawrence DA (2006). Effect of pharmacologic plasminogen activator inhibitor-1 inhibition on cell motility and tumor angiogenesis. *J Thromb Haemost* **4,** 2710-2715.

Leissner P, Verjat T, Bachelot T, Paye M, Krause A, Puisieux A & Mougin B (2006). Prognostic significance of urokinase plasminogen activator and plasminogen activator inhibitor-1 mRNA expression in lymph node- and hormone receptor-positive breast cancer. *BMC Cancer* **6,** 216.

Lempinen M, Inkinen K, Wolff H & Ahonen J (2000). Matrix metalloproteinases 2 and 9 in indomethacin-induced rat gastric ulcer. *Eur Surg Res* **32,** 169-176.

Lester RD, Jo M, Montel V, Takimoto S & Gonias SL (2007). uPAR induces epithelial-mesenchymal transition in hypoxic breast cancer cells. *J Cell Biol* **178,** 425-436.

Leung WK & Sung JJ (2002). Review article: intestinal metaplasia and gastric carcinogenesis. *Aliment Pharmacol Ther* **16,** 1209-1216.

Leung WK, Lin SR, Ching JY, To KF, Ng EK, Chan FK, Lau JY & Sung JJ (2004). Factors predicting progression of gastric intestinal metaplasia: results of a randomised trial on Helicobacter pylori eradication. *Gut* **53,** 1244-1249.

Levy M (1974). Aspirin use in patients with major upper gastrointestinal bleeding and peptic-ulcer disease. A report from the Boston Collaborative Drug Surveillance Program, Boston University Medical Center. *N Engl J Med* **290,** 1158-1162.

Li Q, Karam SM & Gordon JI (1995). Simian virus 40 T antigen-induced amplification of pre-parietal cells in transgenic mice. Effects on other gastric epithelial cell lineages and evidence for a p53-independent apoptotic mechanism that operates in a committed progenitor. *J Biol Chem* **270,** 15777-15788.

Li Q, Karam SM & Gordon JI (1996). Diphtheria toxin ablation of parietal cells in the stomach of transgenic mice. *J Biol Chem* **271,** 3671-3676.

Li Q, Karam SM, Coerver KA, Matzuk MM & Gordon JI (1998). Stimulation of activin receptor II signaling pathways inhibits differentiation of multiple gastric epithelial lineages. *Mol Endocrinol* **12,** 181-192.

Li SD, Kersulyte D, Lindley IJ, Neelam B, Berg DE & Crabtree JE (1999). Multiple genes in the left half of the cag pathogenicity island of Helicobacter pylori are required for tyrosine kinase-dependent transcription of interleukin-8 in gastric epithelial cells. *Infect Immun* **67,** 3893-3899.

Li YS, Milner PG, Chauhan AK, Watson MA, Hoffman RM, Kodner CM, Milbrandt J & Deuel TF (1990). Cloning and expression of a developmentally regulated protein that induces mitogenic and neurite outgrowth activity. *Science* **250,** 1690-1694.

Lichtenberger LM, Dial EJ, Ottlecz A, Romero JJ, Lechago J & Fox JG (1999). Attenuation of hydrophobic phospholipid barrier is an early event in Helicobacter felis-induced gastritis in mice. *Dig Dis Sci* **44,** 108-115.

Lim JH, Woo CH & Li JD (2011). Critical role of type 1 plasminogen activator inhibitor (PAI-1) in early host defense against nontypeable Haemophilus influenzae (NTHi) infection. *Biochem Biophys Res Commun* **414,** 67-72.

Lin CT, Tang HY, Han YS, Liu HP, Huang SF, Chien CH, Shyy J, Chiu JJ & Chen X (2010). Downregulation of Signaling-active IGF-1 by Dipeptidyl Peptidase IV (DPP-IV). *Int J Biomed Sci* **6,** 301-309.

Lin F, Wang N & Zhang TC (2012). The role of endothelial-mesenchymal transition in development and pathological process. *IUBMB Life* **64,** 717-723.

Lin MT, Kuo IH, Chang CC, Chu CY, Chen HY, Lin BR, Sureshbabu M, Shih HJ & Kuo ML (2008). Involvement of hypoxia-inducing factor-1alpha-dependent plasminogen activator inhibitor-1 up-regulation in Cyr61/CCN1-induced gastric cancer cell invasion. *J Biol Chem* **283,** 15807-15815.

Lindberg P, Larsson A & Nielsen BS (2006). Expression of plasminogen activator inhibitor-1, urokinase receptor and laminin gamma-2 chain is an early coordinated event in incipient oral squamous cell carcinoma. *Int J Cancer* **118,** 2948-2956.

Liu D, Aguirre Ghiso J, Estrada Y & Ossowski L (2002). EGFR is a transducer of the urokinase receptor initiated signal that is required for in vivo growth of a human carcinoma. *Cancer Cell* **1,** 445-457.

Liu J, Wang Y, Pan Q, Su Y, Zhang Z, Han J, Zhu X, Tang C & Hu D (2012). Wnt/β-catenin pathway forms a negative feedback loop during TGF-β1 induced human normal skin fibroblast-to-myofibroblast transition. *J Dermatol Sci* **65,** 38-49.

Liu T, Hu B, Choi YY, Chung M, Ullenbruch M, Yu H, Lowe JB & Phan SH (2009). Notch1 signaling in FIZZ1 induction of myofibroblast differentiation. *Am J Pathol* **174,** 1745-1755.

Lu Y, Germano P, Ohning GV, Vu JP & Pisegna JR (2011). PAC1 deficiency in a murine model induces gastric mucosa hypertrophy and higher basal gastric acid output. *J Mol Neurosci* **43,** 76-84.

Lugea A, Mourelle M, Guarner F, Domingo A, Salas A & Malagelada JR (1994). Phosphatidylcholines as mediators of adaptive cytoprotection of the rat duodenum. *Gastroenterology* **107,** 720-727.

Lv ZD, Wang HB, Dong Q, Kong B, Li JG, Yang ZC, Qu HL, Cao WH & Xu HM (2013). Mesothelial cells differentiate into fibroblast-like cells under the scirrhous gastric cancer microenvironment and promote peritoneal carcinomatosis in vitro and in vivo. *Mol Cell Biochem* **377,** 177-185.

Lynch DA, Mapstone NP, Clarke AM, Sobala GM, Jackson P, Morrison L, Dixon MF, Quirke P & Axon AT (1995). Cell proliferation in Helicobacter pylori associated gastritis and the effect of eradication therapy. *Gut* **36,** 346-350.

Lyons RM, Gentry LE, Purchio AF & Moses HL (1990). Mechanism of activation of latent recombinant transforming growth factor beta 1 by plasmin. *J Cell Biol* **110,** 1361-1367.

Madách K, Aladzsity I, Szilágyi A, Fust G, Gál J, Pénzes I & Prohászka Z (2010). 4G/5G polymorphism of PAI-1 gene is associated with multiple organ dysfunction and septic shock in pneumonia induced severe sepsis: prospective, observational, genetic study. *Crit Care* **14,** R79.

Madsen CD, Ferraris GM, Andolfo A, Cunningham O & Sidenius N (2007). uPAR-induced cell adhesion and migration: vitronectin provides the key. *J Cell Biol* **177,** 927-939.

Mahida YR, Beltinger J, Makh S, Göke M, Gray T, Podolsky DK & Hawkey CJ (1997). Adult human colonic subepithelial myofibroblasts express extracellular matrix proteins and cyclooxygenase-1 and -2. *Am J Physiol Gastrointest Liver Physiol* **273,** G1341-G1348.

Mahler M, Janke C, Wagner S & Hedrich HJ (2002). Differential susceptibility of inbred mouse strains to Helicobacter pylori infection. *Scand J Gastroenterol* **37,** 267-278.

Majno G, Gabbiani G, Hirschel BJ, Ryan GB & Statkov PR (1971). Contraction of granulation tissue in vitro: similarity to smooth muscle. *Science* **173,** 548-550.

Makino H, Uetake H, Danenberg K, Danenberg PV & Sugihara K (2008). Efficacy of laser capture microdissection plus RT-PCR technique in analyzing gene expression levels in human gastric cancer and colon cancer. *BMC Cancer* **25,** 210.

Marchetti M, Aricò B, Burroni D, Figura N, Rappuoli R & Ghiara P (1995). Development of a mouse model of *H. pylori* infection that mimics human disease. *Science* **267,** 1655-1658.

Marshall BJ & Warren R (1984). Unidentified curved bacilli in the stomach of patients with gastritis and peptic ulceration. *Lancet* **323,** 1311-1315.

Martin GR & Wallace JL (2006). Gastrointestinal inflammation: a central component of mucosal defense and repair. *Exp Biol Med* **231,** 130-137.

Matarese G, Di Giacomo A, Sanna V, Lord GM, Howard JK, Di Tuoro A, Bloom SR, Lechler RI, Zappacosta S & Fontana S (2001). Requirement for leptin in the induction and progression of autoimmune encephalomyelitis. *J Immunol* **166,** 5909-5916.

Matos JI, de Sousa HA, Marcos-Pinto R & Dinis-Ribeiro M (2013). Helicobacter pylori CagA and VacA genotypes and gastric phenotype: a meta-analysis. *Eur J Gastroenterol Hepatol,* Advance online publication.

Matson CA, Wiater MF, Kuijper JL & Weigle DS (1997). Synergy between leptin and cholecystokinin (CCK) to control daily caloric intake. *Peptides* **18,** 1275-1278.

Matson CA & Ritter RC (1999). Long-term CCK-leptin synergy suggests a role for CCK in the regulation of body weight. *Am J Physiol Regul Integr Comp Physiol* **276,** R1038-R1045.

Matsui H, Nagano Y, Shimokawa O, Kaneko T, Rai K, Udo J, Hirayama A, Nakamura Y, Indo HP, Majima HJ & Hyodo I (2011). Gastric acid induces mitochondrial superoxide production and lipid peroxidation in gastric epithelial cells. *J Gastroenterol* **46,** 1167-1176.

Matsuo S, López-Guisa JM, Cai X, Okamura DM, Alpers CE, Bumgarner RE, Peters MA, Zhang G & Eddy AA (2005). Multifunctionality of PAI-1 in fibrogenesis: evidence from obstructive nephropathy in PAI-1-overexpressing mice. *Kidney Int* **67,** 2221-2238.

Matsuzawa Y (1997). Induction of heparin binding epidermal growth factor-like growth factor and amphiregulin mRNAs by gastrin in the rat stomach. *Biochem Biophys Res Commun* **235,** 520-523.

May AE, Kanse SM, Lund LR, Gisler RH, Imhof BA & Preissner KT (1998) Urokinase receptor (CD87) regulates leukocyte recruitment via beta 2 integrins in vivo. *J Exp Med* **188,** 1029-1037.

McCaig C, Duval C, Hemers E, Steele I, Pritchard DM, Przemeck S, Dimaline R, Ahmed S, Bodger K, Kerrigan DD, Wang TC, Dockray GJ & Varro A (2006). The role of matrix metalloproteinase-7 in redefining the gastric microenvironment in response to Helicobacter pylori. *Gastroenterology* **130,** 1754-1763.

McColl KE, el-Omar EM & Gillen D (1997). The role of H. pylori infection in the pathophysiology of duodenal ulcer disease. *J Physiol Pharmacol* **48,** 287-295.

McMahon GA, Petitclerc E, Stefansson S, Smith E, Wong MK, Westrick RJ, Ginsburg D, Brooks PC & Lawrence DA (2001). Plasminogen activator inhibitor-1 regulates tumor growth and angiogenesis. *J Biol Chem* **276,** 33964-33968.

McNamara D & El-Omar E (2008). *Helicobacter pylori* infection & the pathogenesis of gastric cancer: A paradigm for host-bacterial interactions. *Dig Liver Dis* **40,** 504-509.

Mera R, Fontham ET, Bravo LE, Bravo JC, Piazuelo MB, Camargo MC & Correa P (2005). Long term follow up of patients treated for Helicobacter pylori infection. *Gut* **54,** 1536-1540.

Mercer DW, Cross JM, Smith GS & Miller TA (1997). Protective action of gastrin-17 against alcohol-induced gastric injury in the rat: role in mucosal defense. *Am J Physiol Gastrointest Liver Physiol* **273,** G365-G373.

Mesters RM, Flörke N, Ostermann H & Kienast J (1996). Increase of plasminogen activator inhibitor levels predicts outcome of leukocytopenic patients with sepsis. *Thromb Haemost* **75,** 902-907.

Midgley AC, Rogers M, Hallett MB, Clayton A, Bowen T, Phillips AO & Steadman R (2013). Transforming growth factor-β1 (TGF-β1)-stimulated fibroblast to myofibroblast differentiation is mediated by hyaluronan (HA)-facilitated epidermal growth factor receptor (EGFR) and CD44 co-localization in lipid rafts. *J Biol Chem* **288,** 14824-14838.

Mignatti P & Rifkin DB (1993). Biology and biochemistry of proteinases in tumor invasion. *Physiol Rev* **73,** 161-195.

Mills JC, Andersson N, Hong CV, Stappenbeck TS & Gordon JI (2002). Molecular characterization of mouse gastric epithelial progenitor cells. *Proc Natl Acad Sci U S A* **99,** 14819-14824.

Minowa H, Takahashi Y, Tanaka T, Naganuma K, Ida S, Maki I & Yoshioka A (1999). Four cases of bleeding diathesis in children due to congenital plasminogen activator inhibitor-1 deficiency. *Haemostasis* **29,** 286-291.

Miyazaki Y, Shinomura Y, Tsutsui S, Zushi S, Higashimoto Y, Kanayama S, Higashiyama S, Taniguchi N & Matsuzawa Y (1999). Gastrin induces heparin-binding epidermal growth factor-like growth factor in rat gastric epithelial cells transfected with gastrin receptor. *Gastroenterology* **116,** 78-89.

Mohammadi M, Redline R, Nedrud J & Czinn S (1996). Role of the host in pathogenesis of Helicobacter-associated gastritis: H. felis infection of inbred and congenic mouse strains. *Infect Immun* **64,** 238-245.

Mondino A & Blasi F (2004). uPA and uPAR in fibrinolysis, immunity and pathology. *Trends Immunol* **25,** 450-455.

Monea S, Lehti K, Keski-Oja J & Mignatti P (2002). Plasmin activates pro-matrix metalloproteinase-2 with a membrane-type 1 matrix metalloproteinase-dependent mechanism. *J Cell Physiol* **192,** 160-170.

Morise Z, Granger DN, Fuseler JW, Anderson DC & Grisham MB (1999). Indomethacin induced gastropathy in CD18, intercellular adhesion molecule 1, or P-selectin deficient mice. *Gut* **45,** 523-528.

Moriwaki H, Stempien-Otero A, Kremen M, Cozen AE & Dichek DA (2004). Overexpression of urokinase by macrophages or deficiency of plasminogen activator inhibitor type 1 causes cardiac fibrosis in mice. *Circ Res* **95,** 637-644.

Moss SF, Calam J, Agarwal B, Wang S & Holt PR (1996). Induction of gastric epithelial apoptosis by Helicobacter pylori. *Gut* **38,** 498-501.

Mozsik G, Kiraly A, Garamszegi M, Javor T, Nagy L, Suto G, Toth G & Vincze A (1991). Failure of prostacyclin, β-carotene, atropine and cimetidine to produce gastric cyto- and general mucosal protection in surgically vagotomized rats. *Life Sci* **49,** 1383-1389.

Mozsik G, Kiraly A, Garamszegi M, Javor T, Nagy L, Nemeth A, Suto G & Vincze A (1992). Mechanisms of vagal nerve in gastric mucosal defense: unchanged gastric emptying and increased vascular permeability. *J Clin Gastroenterol* **14 Suppl 1,** S140-S144.

Mozsik G, Karadi O, Kiraly A, Debreceni A, Figler M, Nagy L, Par A, Par G, Suto G & Vincze A (2001). The key-role of vagal nerve and adrenals in the cytoprotection and general gastric mucosal integrity. *J Physiol Paris* **95,** 229-237.

Mukaratirwa S, de Witte E, van Ederen AM & Nederbragt H (2003). Tenascin expression in relation to stromal tumour cells in canine gastrointestinal epithelial tumours. *J Comp Pathol* **129,** 137-146.

Murakami M, Yoo JK, Teramura S, Inada M, Saita H, Matsuo K, Kusaka S, Kita T & Miyake T (1989). Cytoprotective effect of plasmin inhibitor on necrotizing agent-induced gastric lesions in rats. *Jpn J Pharmacol* **50,** 72-74.

Murata-Kamiya N (2011). Pathophysiological functions of the CagA oncoprotein during infection by Helicobacter pylori. *Microbes Infect* **13,** 799-807.

Murayama Y, Miyagawa J, Higashiyama S, Kondo S, Yabu M, Isozaki K, Kayanoki Y, Kanayama S, Shinomura Y, Taniguchi N & Matsuzawa Y (1995). Localization of heparin-binding epidermal growth factor-like growth factor in human gastric mucosa. *Gastroenterology* **109,** 1051-1059.

Mutch DM, Berger A, Mansourian R, Rytz A & Roberts MA (2002). The limit fold change model: a practical approach for selecting differentially expressed genes from microarray data. *BMC Bioinformatics* **21,** 17.

Mutoh H, Ota S, Hiraishi H, Ivey KJ, Terano A & Sugimoto T (1995). Adaptive cytoprotection in cultured rat gastric mucus-producing cells. Role of mucus and prostaglandin synthesis. *Dig Dis Sci* **40,** 872-878.

Nagase H, Enghild JJ, Suzuki K & Salvesen S (1990). Stepwise activation mechanisms of the precursor of matrix metalloproteinase 3 (stromelysin) by proteinases and (4-aminophenyl)mercuric acetate. *Biochem* **29,** 5783-5789.

Nagatsu T, Hino M, Fuyamada H, Hayakawa T, Sakakibara S, Nakagawa Y & Takemoto T (1976). New chromogenic substrates for X-prolyl dipeptidyl-aminopeptidase. *Anal Biochem* **74,** 466-476.

Nakamura K, Rokutan K, Marui N, Aoike A & Kawai K (1991). Induction of heat shock proteins and their implication in protection against ethanol-induced damage in cultured guinea pig gastric mucosal cells. *Gastroenterology* **101,** 161-166.

Nakamura M, Konno H, Tanaka T, Maruo Y, Nishino N, Aoki K, Baba S, Sakaguchi S, Takada Y & Takada A (1992). Possible role of plasminogen activator inhibitor 2 in the prevention of the metastasis of gastric cancer tissues. *Thromb Res* **65,** 709-719.

Nakamura M, Takahashi S, Matsui H, Nishikawa K, Akiba Y & Ishii H (2002). Persistent increase in myofibroblasts in Helicobacter heilmannii-infected mice but not in Helicobacter pylori-infected Mongolian gerbils: colocalization of COX-2 and bFGF immunoreactivity. *Aliment Pharmacol Ther* **16 Suppl 2,** 174-179.

Nam KT, Lee HJ, Mok H, Romero-Gallo J, Crowe JE Jr, Peek RM Jr & Goldenring JR (2009). Amphiregulin-deficient mice develop spasmolytic polypeptide expressing metaplasia and intestinal metaplasia. *Gastroenterology* **136,** 1288-1296.

Necchi V, Candusso ME, Tava F, Luinetti O, Ventura U, Fiocca R, Ricci V & Solcia E (2007) .Intracellular, intercellular, and stromal invasion of gastric mucosa, preneoplastic lesions, and cancer by Helicobacter pylori. *Gastroenterology* **132,** 1009-1023.

Negrini R, Lisato L, Zanella I, Cavazzini L, Gullini S, Villanacci V, Poiesi C, Albertini A & Ghielmi S (1991). Helicobacter pylori infection induces antibodies cross-reacting with human gastric mucosa. *Gastroenterology* **101,** 437-445.

Negrini R, Savio A & Appelmelk BJ (1997). Autoantibodies to gastric mucosa in Helicobacter pylori infection. *Helicobacter* **2 Suppl 1,** S13-S16.

Nekarda H, Schmitt M, Ulm K, Wenninger A, Vogelsang H, Becket K, Roder JD, Fink U & Siewert JR (1994). Prognostic impact of urokinase-type plasminogen activator and its inhibitor PAI-1 in completely resected gastric cancer. *Can Res* **54,** 2900-2907.

Németh IB & Tiszlavicz L (2012). Biphenotypic surface epithelial cells in the gastrointestinal tube with mixed epithelial-myofibroblastic differentiation: a paradigm. *Pathol Oncol Res* **18,** 391-396.

Nguyen DH, Hussaini IM & Gonias SL (1998). Binding of urokinase-type plasminogen activator to its receptor in MCF-7 cells activates extracellular signal-regulated kinase 1 and 2 which is required for increased cellular motility. *J Biol Chem* **273,** 8502-8507.

Nguyen DH, Webb DJ, Catling AD, Song Q, Dhakephalkar A, Weber MJ, Ravichandran KS & Gonias SL (2000). Urokinase-type plasminogen activator stimulates the Ras/Extracellular signal-regulated kinase (ERK) signaling pathway and MCF-7 cell migration by a mechanism that requires focal adhesion kinase, Src, and Shc. Rapid dissociation of GRB2/Sps-Shc complex is associated with the transient phosphorylation of ERK in urokinase-treated cells. *J Biol Chem* **275,** 19382-19388.

Nielsen BS, Rank F, Illemann M, Lund LR & Danø K (2007). Stromal cells associated with early invasive foci in human mammary ductal carcinoma in situ coexpress urokinase and urokinase receptor. *Int J Cancer* **120,** 2086-2095.

Nielsen LS, Hansen JG, Skriver L, Wilson EL, Kaltoft K, Zeuthen J & Danø K (1982). Purification of zymogen to plasminogen activator from human glioblastoma cells by affinity chromatography with monoclonal antibody. *Biochemistry* **21,** 6410-6415.

Nilsson IM, Bergentz SE, Hedner U & Kullenberg K (1975). Gastric fibrinolysis. *Thromb Diath Haemorrh* **34,** 409-418.

Nishida T, Tsuji S, Kimura A, Tsujii M, Ishii S, Yoshio T, Shinzaki S, Egawa S, Irie T, Yasumaru M, Iijima H, Murata H, Kawano S & Hayashi N (2006). Endothelin-1, an ulcer inducer, promotes gastric ulcer healing via mobilizing gastric myofibroblasts and stimulates production of stroma-derived factors. *Am J Physiol Gastrointest Liver Physiol* **290,** G1041-G1050.

Nishioka N, Matsuoka T, Yashiro M, Hirakawa K, Olden K & Roberts JD (2012). Plasminogen activator inhibitor 1 RNAi suppresses gastric cancer metastasis in vivo. *Cancer Sci* **103,** 228-232.

Nomura S, Baxter T, Yamaguchi H, Leys C, Vartapetian AB, Fox JG, Lee JR, Wang TC, & Goldenring JR (2004). Spasmolytic polypeptide expressing metaplasia to preneoplasia in H. felis-infected mice. *Gastroenterology* **127,** 582-594.

Norsett KG, Steele I, Duval C, Sammut SJ, Murugesan SV, Kenny S, Rainbow L, Dimaline R, Dockray GJ, Pritchard DM & Varro A (2011). Gastrin stimulates expression of plasminogen activator inhibitor-1 in gastric epithelial cells. *Am J Physiol Gastrointest Liver Physiol* **301,** G446-G453.

Nykjaer A, Møller B, Todd RF 3rd, Christensen T, Andreasen PA, Gliemann J & Petersen CM (1994). Urokinase receptor. An activation antigen in human T lymphocytes. *J Immunol* **152,** 505-516.

Oda T, Jung YO, Kim HS, Cai X, López-Guisa JM, Ikeda Y & Eddy AA (2001). PAI-1 deficiency attenuates the fibrogenic response to ureteral obstruction. *Kidney Int* **60,** 587-596.

Odenbreit S, Püls J, Sedlmaier B, Gerland E, Fischer W & Haas R (2000). Translocation of Helicobacter pylori CagA into gastric epithelial cells by type IV secretion. *Science* **287,** 1497-1500.

Offersen BV, Nielsen BS, Høyer-Hansen G, Rank F, Hamilton-Dutoit S, Overgaard J & Andreasen PA (2003). The myofibroblast is the predominant plasminogen activator inhibitor-1 expressing cell type in human breast cancer. *Am J Pathol* **163,** 1887-1899.

Ogawa M, Nomura S, Varro A, Wang TC, Goldenring JR (2006). Altered metaplastic response of waved-2 EGF receptor mutant mice to acute oxyntic atrophy. *Am J Physiol Gastrointest Liver Physiol* **290,** G793-G804.

Oh JD, Karam SM & Gordon JI (2005). Intracellular Helicobacter pylori in gastric epithelial progenitors. *Proc Natl Acad Sci U S A* **102,** 5186-5191.

Ohning GV, Wong HC, Lloyd KC & Walsh JH (1996). Gastrin mediates the gastric mucosal proliferative response to feeding. *Am J Physiol Gastrointest Liver Physiol* **271,** G470-G476.

Ohnishi N, Yuasa H, Tanaka S, Sawa H, Miura M, Matsui A, Higashi H, Musashi M, Iwabuchi K, Suzuki M, Yamada G, Azuma T & Hatakeyama M (2008). Transgenic expression of Helicobacter pylori CagA induces gastrointestinal and hematopoietic neoplasms in mouse. *Proc Natl Acad Sci U S A* **105,** 1003-1008.

Okada H, Inoue T, Kanno Y, Kobayashi T, Watanabe Y, Ban S, Neilson EG & Suzuki H (2003). Selective depletion of fibroblasts preserves morphology and the functional integrity of peritoneum in transgenic mice with peritoneal fibrosing syndrome. *Kidney Int* **64,** 1722-1732.

Okamoto R, Yajima T, Yamazaki M, Kanai T, Mukai M, Okamoto S, Ikeda Y, Hibi T, Inazawa J & Watanabe M (2002). Damaged epithelia regenerated by bone marrow-derived cells in the human gastrointestinal tract. *Nat Med* **8,** 1011-1017.

Orihata M, Watanabe Y, Tanaka M, Okubo T & Sakakibara N (1989). The relationship between adaptive cytoprotection and prostaglandin contents in the rat stomachs after oral administration of 0.35N HCl. *Scand J Gastroenterol Suppl* **162,** 79-82.

Orsini B, Calabrò A, Milani S, Grappone C, Herbst H & Surrenti C (1993). Localization of epidermal growth factor/transforming growth factor-alpha receptor in the human gastric mucosa. An immunohistochemical and in situ hybridization study. *Virchows Arch A Pathol Anat Histopathol* **423,** 57-63.

Pagliocca A, Hegyi P, Venglovecz V, Rackstraw SA, Khan Z, Burdyga G, Wang TC, Dimaline R, Varro A & Dockray GJ (2008). Identification of ezrin as a target of gastrin in immature mouse gastric parietal cells. *Exp Physiol* **93,** 1174-1189.

Pai R, Szabo IL, Giap AQ, Kawanaka H & Tarnawski AS (2001). Nonsteroidal anti-inflammatory drugs inhibit re-epithelialization of wounded gastric monolayers by interfering with actin, Src, FAK, and tensin signaling. *Life Sci* **69,** 3055-3071.

Pai R, Soreghan B, Szabo IL, Pavelka M, Baatar D & Tarnawski AS (2002). Prostaglandin E2 transactivates EGF receptor: a novel mechanism for promoting colon cancer growth and gastrointestinal hypertrophy. *Nat Med* **8,** 289-293.

Palframan SL, Kwok T & Gabriel K (2012). Vacuolating cytotoxin A (VacA), a key toxin for Helicobacter pylori pathogenesis. Front *Cell Infect Microbiol* **2,** 92.

Park JE, Lenter MC, Zimmermann RN, Garin-Chesa P, Old LJ & Rettig WJ (1999). Fibroblast activation protein, a dual specificity serine protease expressed in reactive human tumor stromal fibroblasts. *J Biol Chem* **274,** 36505-36512.

Park JH, Seok SH, Baek MW, Lee HY, Kim DJ & Park JH (2008). Gastric lesions and immune responses caused by long-term infection with Helicobacter heilmannii in C57BL/6 mice. *J Comp Pathol* **139,** 208-217.

Park JS, Park JH, Khoi PN, Joo YE & Jung YD (2011). MSP-induced RON activation upregulates uPAR expression and cell invasiveness via MAPK, AP-1 and NF-kappaB signals in gastric cancer cells. *Carcinogenesis* **32,** 175-181.

Parkin DM, Bray F, Ferlay J & Pisani P (2005). Global cancer statistics, 2002. *CA Cancer J Clin* **55,** 74-108.

Parsonnet J, Friedman GD, Orentreich N & Vogelman H (1997). Risk for gastric cancer in people with CagA positive or CagA negative Helicobacter pylori infection. *Gut* **40,** 297-301.

Peek RM Jr & Blaser MJ (2002). Helicobacter pylori and gastrointestinal tract adenocarcinomas. *Nat Rev Cancer* **2,** 28-37.

Peek RM Jr & Crabtree JE (2006). Helicobacter infection and gastric neoplasia. *J Pathol* **208,** 233-248.

Pepper SD, Saunders EK, Edwards LE, Wilson CL & Miller CJ (2007). The utility of MAS5 expression summary and detection call algorithms. *BMC Bioinformatics* **30,** 273.

Peskar BM, Maricic N, Gretzera B, Schuligoi R & Schmassmann A (2001). Role of cyclooxygenase-2 in gastric mucosal defense. *Life Sci* **69,** 2993-3003.

Philpott DJ, Belaid D, Troubadour P, Thiberge JM, Tankovic J, Labigne A & Ferrero RL (2002) Reduced activation of inflammatory responses in host cells by mouse-adapted *Helicobacter pylori* isolates. *Cell Microbiol* **4,** 285-296.

Piera-Velazquez S, Li Z & Jimenez SA (2011). Role of endothelial-mesenchymal transition (EndoMT) in the pathogenesis of fibrotic disorders. *Am J Pathol* **179,** 1074-1080.

Pinchuk IV, Mifflin RC, Saada JI & Powell DW (2010). Intestinal mesenchymal cells. *Curr Gastroenterol Rep* **12,** 310-318.

Pinchuk IV, Morris KT, Nofchissey RA, Earley RB, Wu JY, Ma TY & Beswick EJ (2013). Stromal cells induce Th17 during Helicobacter pylori infection and in the gastric tumor microenvironment. *PLoS One* **8,** e53798.

Piotrowski J, Piotrowski E, Skrodzka D, Slomiany A & Slomiany BL (1997). Gastric mucosal apoptosis induced by ethanol: effect of antiulcer agents. *Biochem Mol Biol Int* **42,** 247-254.

Pirazzoli V, Ferraris GM & Sidenius N (2013). Direct evidence of the importance of vitronectin and its interaction with the urokinase receptor in tumor growth. *Blood* **121,** 2316-2323.

Plebani M, Herszènyi L, Carraro P, De Paoli M, Roveroni G, Cardin R, Tulassay Z, Naccarato R & Farinati F (1997). Urokinase-type plasminogen activator receptor in gastric cancer: tissue expression and prognostic role. *Clin Exp Metastasis* **15,** 418-425.

Plesner, T, Ralfkiaer E, Wittrup M, Johnsen H, Pyke C, Pedersen TL, Hansen NE & Danø K (1994). Expression of the receptor for urokinase-type plasminogen activator in normal and neoplastic blood cells and hematopoietic tissue. *Am J Clin Pathol* **102,** 835-841.

Ploplis VA, Balsara R, Sandoval-Cooper MJ, Yin ZJ, Batten J, Modi N, Gadoua D, Donahue D, Martin JA & Castellino FJ (2004). Enhanced in vitro proliferation of aortic endothelial cells from plasminogen activator inhibitor-1-deficient mice. *J Biol Chem* **279,** 6143-6151.

Ploug M, Rønne E, Behrendt N, Jensen AL, Blasi F & Danø K (1991). Cellular receptor for urokinase plasminogen activator. Carboxyl-terminal processing and membrane anchoring by glycosylphosphatidylinositol. *J Biol Chem* **266,** 1926-1933.

Powell DW, Mifflin RC, Valentich JD, Crowe SE, Saada JI & West AB (1999). Myofibroblasts. I. Paracrine cells important in health and disease. *Am J Physiol Cell Physiol* **277,** C1-C19.

Powell DW, Adegboyega PA, Di Mari JF & Mifflin RC (2005). Epithelial cells and their neighbors I. Role of intestinal myofibroblasts in development, repair, and cancer. *Am J Physiol Gastrointest Liver Physiol* **289,** G2-G7.

Powell DW, Pinchuk IV, Saada JI, Chen X & Mifflin RC (2011). Mesenchymal cells of the intestinal lamina propria. *Annu Rev Physiol* **73,** 213-237.

Pradeepkumar Singh L, Vivek Sharma A & Swarnakar S (2011). Upregulation of collagenase-1 and -3 in indomethacin-induced gastric ulcer in diabetic rats: role of melatonin. *J Pineal Res* **51,** 61-74.

Pritchard DM & Przemeck SM (2004). Review article: How useful are the rodent animal models of gastric adenocarcinoma? *Aliment Pharmacol Ther* **19,** 841-859.

Pritchard DM, Berry D, Przemeck SM, Campbell F, Edwards SW & Varro A (2008). Gastrin increases mcl-1 expression in type I gastric carcinoid tumors and a gastric epithelial cell line that expresses the CCK-2 receptor. *Am J Physiol Gastrointest Liver Physiol* **295,** G798-G805.

Przemeck SM, Duckworth CA & Pritchard DM (2007). Radiation-induced gastric epithelial apoptosis occurs in the proliferative zone and is regulated by p53, bak, bax, and bcl-2. *Am J Physiol Gastrointest Liver Physiol* **292,** G620-G627.

Przemeck SM, Varro A, Berry D, Steele I, Wang TC, Dockray GJ & Pritchard DM (2008). Hypergastrinemia increases gastric epithelial susceptibility to apoptosis. *Regul Pept* **146,** 147-156.

Qiao XT, Ziel JW, McKimpson W, Madison BB, Todisco A, Merchant JL, Samuelson LC & Gumucio DL (2007). Prospective identification of a multilineage progenitor in murine stomach epithelium. *Gastroenterology* **133,** 1989-1998.

Quante M, Tu SP, Tomita H, Gonda T, Wang SS, Takashi S, Baik GH, Shibata W, Diprete B, Betz KS, Friedman R, Varro A, Tycko B & Wang TC (2011). Bone marrow-derived myofibroblasts contribute to the mesenchymal stem cell niche and promote tumor growth. *Cancer Cell* **19,** 257-272.

Quax PH, van den Hoogen CM, Verheijen JH, Padro T, Zeheb R, Gelehrter TD, van Berkel TJ, Kuiper J & Emeis JJ (1990). Endotoxin induction of plasminogen activator and plasminogen activator inhibitor type 1 mRNA in rat tissues in vivo. *J Biol Chem* **265,** 15560-15563.

Quinn SJ, Bai M & Brown EM (2004). pH Sensing by the calcium-sensing receptor. *J Biol Chem* **279,** 37241-37249.

Raaphorst J, Johan Groeneveld AB, Bossink AW & Erik Hack C (2001). Early inhibition of activated fibrinolysis predicts microbial infection, shock and mortality in febrile medical patients. *Thromb Haemost* **86,** 543-549.

Radulescu S, Ridgway RA, Cordero J, Athineos D, Salgueiro P, Poulsom R, Neumann J, Jung A, Patel S, Woodgett J, Barker N, Pritchard DM, Oien K & Sansom OJ (2013). Acute WNT signalling activation perturbs differentiation within the adult stomach and rapidly leads to tumour formation. *Oncogene* **32,** 2048-2057.

Rakic JM, Maillard CM, Jost M, Bajou K, Masson V, Devy L, Lambert V, Foidart JM & Noël A (2003). Role of plasminogen activator-plasmin system in tumor angiogenesis. *Cell Mol Life Sci* **60,** 463-473.

Ramalho-Santos M, Melton DA & McMahon AP (2000). Hedgehog signals regulate multiple aspects of gastrointestinal development. *Development* **127,** 2763-2772.

Rauvala H (1989). An 18-kd heparin-binding protein of developing brain that is distinct from fibroblast growth factors. *EMBO J* **8,** 2933-2941.

Reimann F, Tolhurst G & Gribble FM (2012). G-protein-coupled receptors in intestinal chemosensation. *Cell Metab* **15,** 421-431.

Reis CA, David L, Correa P, Carneiro F, de Bolós C, Garcia E, Mandel U, Clausen H & Sobrinho-Simões M (1999). Intestinal metaplasia of human stomach displays distinct patterns of mucin (MUC1, MUC2, MUC5AC, and MUC6) expression. *Cancer Res* **59,** 1003-1007.

Renckens R, Roelofs JJ, De Waard V, Florquin S, Lijnen HR, Carmeliet P & van der Poll T (2005). The role of plasminogen activator inhibitor type 1 in the inflammatory response to local tissue injury. *J Thromb Haemost* **3,** 1018-1025.

Renckens R, Pater JM & van der Poll T (2006). Plasminogen activator inhibitor type-1-deficient mice have an enhanced IFN-gamma response to lipopolysaccharide and staphylococcal enterotoxin *B J Immunol* **177,** 8171-8176.

Renckens R, Roelofs JJ, Bonta PI, Florquin S, de Vries CJ, Levi M, Carmeliet P, van't Veer C & van der Poll T (2007). Plasminogen activator inhibitor type 1 is protective during severe Gram-negative pneumonia. *Blood* **109,** 1593-1601.

Resnati M, Pallavicini I, Wang JM, Oppenheim J, Serhan CN, Romano M & Blasi F (2002). The fibrinolytic receptor for urokinase activates the G protein-coupled chemotactic receptor FPRL1/LXA4R. *Proc Natl Acad Sci U S A* **99,** 1359-1364.

Resnick MB, Sabo E, Meitner PA, Kim SS, Cho Y, Kim HK, Tavares R & Moss SF (2006). Global analysis of the human gastric epithelial transcriptome altered by Helicobacter pylori eradication in vivo. *Gut* **55,** 1717-1724.

Rhead JL, Letley DP, Mohammadi M, Hussein N, Mohagheghi MA, Eshagh Hosseini M & Atherton JC (2007). A new Helicobacter pylori vacuolating cytotoxin determinant, the intermediate region, is associated with gastric cancer. *Gastroenterology* **133,** 926-936.

Rieder G, Fischer W & Haas R (2005). Interaction of Helicobacter pylori with host cells: function of secreted and translocated molecules. *Curr Opin Microbiol* **8,** 67-73.

Rijneveld AW, Levi M, Florquin S, Speelman P, Carmeliet P & van Der Poll (2002). Urokinase receptor is necessary for adequate host defense against pneumococcal pneumonia. *J Immunol* **168,** 3507-3511.

Rijneveld AW, Florquin S, Bresser P, Levi M, De Waard V, Lijnen R, Van Der Zee JS, Speelman P, Carmeliet P & Van Der Poll T (2003). Plasminogen activator inhibitor type-1 deficiency does not influence the outcome of murine pneumococcal pneumonia. *Blood* **102,** 934-939.

Rindi G, Necchi V, Savio A, Torsello A, Zoli M, Locatelli V, Raimondo F, Cocchi D & Solcia E (2002). Characterisation of gastric ghrelin cells in man and other mammals: studies in adult and fetal tissues. *Histochem Cell Biol* **117,** 511-519.

Rindi G, Leiter AB, Kopin AS, Bordi C & Solcia E (2004). The “normal” endocrine cell of the gut: changing concepts and new evidences. *Ann N Y Acad Sci* **1014,** 1-12.

Robbins KC, Summaria L, Hsich B & Shah RJ (1967). The peptide chains of human plasmin. Mechanism of activation of human plasminogen to plasmin. *J Biol Chem* **242,** 2333-2342.

Robert A, Nezamis JE, Lancaster C & Hanchar AJ (1979). Cytoprotection by prostaglandins in rats. Prevention of gastric necrosis produced by alcohol, HCl, NaOH, hypertonic NaCl, and thermal injury. *Gastroenterology* **77,** 433-443.

Robert A, Nezamis JE, Lancaster C, Davis JP, Field SO & Hanchar AJ (1983). Mild irritants prevent gastric necrosis through “adaptive cytoprotection” mediated by prostaglandins. *Am J Physiol Gastrointest Liver Physiol* **245,** G113-G121.

Robinson K, Argent RH & Atherton JC (2007). The inflammatory and immune response to Helicobacter pylori infection. *Best Pract Res Clin Gastroenterol* **21,** 237-259.

Roca C, Primo L, Valdembri D, Cividalli A, Declerck P, Carmeliet P, Gabriele P & Bussolino F (2003). Hyperthermia inhibits angiogenesis by a plasminogen activator inhibitor 1-dependent mechanism. *Cancer Res* **63,** 1500-1507.

Rodrigues S, Van Aken E, Van Bocxlaer S, Attoub S, Nguyen QD, Bruyneel E, Westley BR, May FE, Thim L, Mareel M, Gespach C & Emami S (2003). Trefoil peptides as proangiogenic factors in vivo and in vitro: implication of cyclooxygenase-2 and EGF receptor signaling. *FASEB J* **17,** 7-16.

Rogers AB, Taylor NS, Whary MT, Stefanich ED, Wang TC & Fox JG (2005). Helicobacter pylori but not high salt induces gastric intraepithelial neoplasia in B6129 mice. *Cancer Res* **65,** 10709-10715.

Rømer MU, Larsen L, Offenberg H, Brünner N & Lademann UA (2008). Plasminogen activator inhibitor 1 protects fibrosarcoma cells from etoposide-induced apoptosis through activation of the PI3K/Akt cell survival pathway. *Neoplasia* **10,** 1083-1091.

Romero-Gallo J, Harris EJ, Krishna U, Washington MK, Perez-Perez GI & Peek RM (2008). Effect of Helicobacter pylori eradication on gastric carcinogenesis. *Lab Invest* **88,** 328-336.

Roschke AV, Tonon G, Gehlhaus KS, McTyre N, Bussey KJ, Lababidi S, Scudiero DA, Weinstein JN & Kirsch IR (2003). Karyotypic complexity of the NCI-60 drug-screening panel. *Cancer Res* **63,** 8634-8547.

Ross DT, Scherf U, Eisen MB, Perou CM, Rees C, Spellman P, Iyer V, Jeffrey SS, Van de Rijn M, Waltham M, Pergamenschikov A, Lee JC, Lashkari D, Shalon D, Myers TG, Weinstein JN, Botstein D & Brown PO (2000). Systematic variation in gene expression patterns in human cancer cell lines. *Nat Genet* **24,** 227-235.

Roth GJ, Stanford N & Majerus PW (1975). Acetylation of prostaglandin synthase by aspirin. *Proc Natl Acad Sci U S A* **72,** 3073-3076.

Roth KA, Kapadia SB, Martin SM & Lorenz RG (1999). Cellular immune responses are essential for the development of Helicobacter felis-associated gastric pathology. *J Immunol* **163,** 1490-1497.

Rothenbacher D & Brenner H (2003). Burden of Helicobacter pylori and H. pylori-related diseases in developed countries: recent developments and future implications. *Microbes Infect* **5,** 693-703.

Ryberg B, Axelson J, Håkanson R, Sundler F, & Mattsson H (1990a). Trophic effects of continuous infusion of [Leu15]-gastrin-17 in the rat. *Gastroenterology* **98,** 33-38.

Ryberg B, Tielemans Y, Axelson J, Carlsson E, Håkanson R, Mattson H, Sundler F & Willems G (1990b). Gastrin stimulates the self-replication rate of enterochromaffinlike cells in the rat stomach. Effects of omeprazole, ranitidine, and gastrin-17 in intact and antrectomized rats. *Gastroenterology* **99,** 935-942.

Sakagami T, Dixon M, O'Rourke J, Howlett R, Alderuccio F, Vella J, Shimoyama T & Lee A (1996). Atrophic gastric changes in both *Helicobacter felis* and *Helicobacter pylori* infected mice are host dependent and separate from antral gastritis. *Gut* **39,** 639-648.

Salama NR, Hartung ML & Müller A (2013). Life in the human stomach: persistence strategies of the bacterial pathogen Helicobacter pylori. *Nat Rev Microbiol* **11,** 385-399.

Salgado S, Garcia J, Vera J, Siller F, Bueno M, Miranda A, Segura A, Grijalva G, Segura J, Orozco H, Hernandez-Pando R, Fafutis M, Aguilar LK, Aguilar-Cordova E & Armendariz-Borunda J (2000). Liver cirrhosis is reverted by urokinase-type plasminogen activator gene therapy. *Mol Ther* **2,** 545-551.

Salic A & Mitchison TJ (2008). A chemical method for fast and sensitive detection of DNA synthesis in vivo. *Proc Natl Acad Sci U S A* **105,** 2415-2420.

Samad F, Yamamoto K & Loskutoff DJ (1996) Distribution and regulation of plasminogen activator inhibitor-1 in murine adipose tissue in vivo. Induction by tumor necrosis factor-alpha and lipopolysaccharide. *J Clin Invest* **97,** 37-46.

Sandberg R & Ernberg I (2005). The molecular portrait of in vitro growth by meta-analysis of gene-expression profiles. *Genome Biol* **6,** R65.

Sandbo N, Ngam C, Torr E, Kregel S, Kach J & Dulin N (2013). Control of myofibroblast differentiation by microtubule dynamics through a regulated localization of mDia2. *J Biol Chem* **288,** 15466-15473.

Sayi A, Kohler E, Hitzler I, Arnold I, Schwendener R, Rehrauer H & Müller A (2009). The CD4+ T cell-mediated IFN-gamma response to Helicobacter infection is essential for clearance and determines gastric cancer risk. *J Immunol* **182,** 7085-7101.

Schistosomes, liver flukes and *Helicobacter pylori.* IARC monographs on the evaluation of carcinogenic risks to humans (1994). *Lyon France: IARC* **61,** 177-241.

Schleef RR, Higgins DL, Pillemer E & Levitt LJ (1989). Bleeding diathesis due to decreased functional activity of type 1 plasminogen activator inhibitor. *J Clin Invest* **83,** 1747-1752.

Schmausser B, Andrulis M, Endrich S, Lee SK, Josenhans C, Müller-Hermelink HK & Eck M (2004). Expression and subcellular distribution of toll-like receptors TLR4, TLR5 and TLR9 on the gastric epithelium in Helicobacter pylori infection. *Clin Exp Immunol* **136,** 521-526.

Schmitz F, Göke MN, Otte JM, Schrader H, Reimann B, Kruse ML, Siegel EG, Peters J, Herzig KH, Fölsch UR & Schmidt WE (2001). Cellular expression of CCK-A and CCK-B/gastrin receptors in human gastric mucosa. *Regul Pept* **102,** 101-110.

Schmitz JM, Durham CG, Schoeb TR, Soltau TD, Wolf KJ, Tanner SM, McCracken VJ & Lorenz RG (2011). Helicobacter felis-associated gastric disease in microbiota-restricted mice. *J Histochem Cytochem* **59,** 826-841.

Schreiber S, Konradt M, Groll C, Scheid P, Hanauer G, Werling HO, Josenhans C & Suerbaum S (2004). The spatial orientation of Helicobacter pylori in the gastric mucus. *Proc Natl Acad Sci U S A* **101,** 5024-5029.

Schubert ML & Peura DA (2008). Control of gastric acid secretion in health and disease. *Gastroenterology* **134,** 1842-1860.

Schwartz GJ, McHugh PR & Moran TH (1993). Gastric loads and cholecystokinin synergistically stimulate rat gastric vagal afferents. *Am J Physiol Regul Integr Comp Physiol* **265,** R872-R876.

Segal ED, Cha J, Lo J, Falkow S & Tompkins LS (1999). Altered states: involvement of phosphorylated CagA in the induction of host cellular growth changes by Helicobacter pylori. *Proc Natl Acad Sci U S A* **96,** 14559-14564.

Seiffert D & Loskutoff DJ (1991). Kinetic analysis of the interaction between type 1 plasminogen activator inhibitor and vitronectin and evidence that the bovine inhibitor binds to a thrombin-derived amino-terminal fragment of bovine vitronectin. *Biochim Biophys Acta* **1078,** 23-30.

Sejima T, Madoiwa S, Mimuro J, Sugo T, Okada K, Ueshima S, Matsuo O, Ishida T, Ichimura K & Sakata Y (2005). Protection of plasminogen activator inhibitor-1-deficient mice from nasal allergy. *J Immunol* **174,** 8135-8143.

Senoo T, Hattori N, Tanimoto T, Furonaka M, Ishikawa N, Fujitaka K, Haruta Y, Murai H, Yokoyama A & Kohno N (2010). Suppression of plasminogen activator inhibitor-1 by RNA interference attenuates pulmonary fibrosis. *Thorax* **65,** 334-340.

Serini G & Gabbiani G (1999). Mechanisms of myofibroblast activity and phenotypic modulation. *Exp Cell Res* **250**, 273-283.

Sharma AV, Ganguly K, Paul S, Maulik N & Swarnakar S (2012). Curcumin heals indomethacin-induced gastric ulceration by stimulation of angiogenesis and restitution of collagen fibers via VEGF and MMP-2 mediated signaling. *Antioxid Redox Signal* **16,** 351-362.

Sharp R, Babyatsky MW, Takagi H, Tågerud S, Wang TC, Bockman DE, Brand SJ & Merlino G (1995). Transforming growth factor alpha disrupts the normal program of cellular differentiation in the gastric mucosa of transgenic mice. *Development* **121,** 149-161.

Shimizu M, Hara A, Okuno M, Matsuno H, Okada K, Ueshima S, Matsuo O, Niwa M, Akita K, Yamada Y, Yoshimi N, Uematsu T, Kojima S, Friedman SL, Moriwaki H & Mori H (2001). Mechanism of retarded liver regeneration in plasminogen activator-deficient mice: impaired activation of hepatocyte growth factor after Fas-mediated massive hepatic apoptosis. *Hepatology* **33,** 569-576.

Shinohara M, Mao M, Keeley TM, El-Zaatari M, Lee HJ, Eaton KA, Samuelson LC, Merchant JL, Goldenring JR & Todisco A (2010). Bone morphogenetic protein signaling regulates gastric epithelial cell development and proliferation in mice. *Gastroenterology* **139,** 2050-2060.

Shubeita HE, Cottey TL, Franke AE & Gerard RD (1990). Mutational and immunochemical analysis of plasminogen activator inhibitor 1. *J Biol Chem* **265,** 18379-18785.

Shujaa N, Zadori ZS, Ronai AZ, Barna I, Mergl Z, Mozes MM & Gyires K (2009). Analysis of the effect of neuropeptides and cannabinoids in gastric mucosal defense initiated centrally in the rat. *J Physiol Pharmacol* **60 Suppl 7,** 93-100.

Sidenius N, Andolfo A, Fesce R & Blasi F (2002). Urokinase regulates vitronectin binding by controlling urokinase receptor oligomerization. *J Biol Chem* **277,** 27982-27990.

Silva E, Teixeira A, David L, Carneiro F, Reis CA, Sobrinho-Simões J, Serpa J, Veerman E, Bolscher J & Sobrinho-Simões M (2002). Mucins as key molecules for the classification of intestinal metaplasia of the stomach. *Virchows Arch* **440,** 311-317.

Simonsson M, Eriksson S, Håkanson R, Lind T, Lönroth H, Lundell L, O'Connor DT & Sundler F (1988). Endocrine cells in the human oxyntic mucosa. A histochemical study. *Scand J Gastroenterol* **23,** 1089-1099.

Singh LP, Mishra A, Saha D & Swarnakar S (2011). Doxycycline blocks gastric ulcer by regulating matrix metalloproteinase-2 activity and oxidative stress. *World J Gastroenterol* **17,** 3310-3321.

Skriver L, Nielsen LS, Stephens R & Danø K (1982). Plasminogen activator released as inactive proenzyme from murine cells transformed by sarcoma virus. *Eur J Biochem* **124,** 409-414.

Slomiany BL, Piotrowski J & Slomiany A (1997). Induction of tumor necrosis factor-alpha and apoptosis in gastric mucosal injury by indomethacin: effect of omeprazole and ebrotidine. *Scand J Gastroenterol* **32,** 638-642.

Smith HW & Marshall CJ (2010). Regulation of cell signalling by uPAR. *Nat Rev Mol Cell Biol* **11,** 23-36.

Soeda S, Oda M, Ochiai T & Shimeno H (2001). Deficient release of plasminogen activator inhibitor-1 from astrocytes triggers apoptosis in neuronal cells. *Brain Res Mol Brain Res* **91,** 96-103.

Soeda S, Shinomiya K, Ochiai T, Koyanagi S, Toda A, Eyanagi R & Shimeno H (2006). Plasminogen activator inhibitor-1 aids nerve growth factor-induced differentiation and survival of pheochromocytoma cells by activating both the extracellular signal-regulated kinase and c-Jun pathways. *Neuroscience* **141,** 101-108.

Soff GA, Sanderowitz J, Gately S, Verrusio E, Weiss I, Brem S & Kwaan HC (1995). Expression of plasminogen activator inhibitor type 1 by human prostate carcinoma cells inhibits primary tumor growth, tumor-associated angiogenesis, and metastasis to lung and liver in an athymic mouse model. *J Clin Invest* **96,** 2593-2600.

Somerville K, Faulkner G & Langman M (1986). Non-steroidal anti-inflammatory drugs and bleeding peptic ulcer. *Lancet* **327,** 462-464.

Steele IA, Dimaline R, Pritchard DM, Peek RM Jr, Wang TC, Dockray GJ & Varro A (2007). Helicobacter and gastrin stimulate Reg1 expression in gastric epithelial cells through distinct promoter elements. *Am J Physiol Gastrointest Liver Physiol* **293,** G347-G354.

Steenfos HH & Jansson JO (1992). Gene expression of insulin-like growth factor-I and IGF-I receptor during wound healing in rats. *Eur J Surg* **158,** 327-331.

Stefansson S & Lawrence DA (1996). The serpin PAI-1 inhibits cell migration by blocking integrin $\alpha_V\beta_3$ binding to vitronectin. *Nature* **383**, 441-443.

Stefansson S, Muhammad S, Cheng XF, Battey FD, Strickland DK & Lawrence DA (1998). Plasminogen activator inhibitor-1 contains a cryptic high affinity binding site for the low density lipoprotein receptor-related protein. *J Biol Chem* **273,** 6358-6366.

Stenberg B, Bylock A & Risberg B (1983a). Local fibrinolytic activity and its structural basis in experimental gastric ulcers. *Eur J Clin Invest* **13,** 297-300.

Stenberg B, Karlsson L, Alpsten M & Risberg B (1983b). Reduction of gastric haemorrhage by fibrinolysis inhibition – an experimental study in rats. *Thromb Haemost* **49,** 106-108.

Stroff T, Plate S, Respondek M, Müller KM & Peskar BM (1995). Protection by gastrin in the rat stomach involves afferent neurons, calcitonin gene-related peptide, and nitric oxide. *Gastroenterology* **109,** 89-97.

Sung CO, Lee KW, Han S & Kim SH (2011). Twist1 is up-regulated in gastric cancer-associated fibroblasts with poor clinical outcomes. *Am J Pathol* **179,** 1827-1838.

Suto G, Kiraly A, Vincze A & Mozsik G (1992). Effect of acute surgical vagotomy on the mucosal content of 6-keto-PGF1 alpha, PGE2 and glutathione after intragastric 96% ethanol treatment in rats. *Acta Physiol Hung* **80,** 205-211.

Suzuki J, Ogawa M, Muto S, Yamaguchi Y, Itai A & Isobe M (2008). The effects of pharmacological PAI-1 inhibition on thrombus formation and neointima formation after arterial injury. *Expert Opin Ther Targets* **12,** 783-794.

Swiercz R, Wolfe JD, Zaher A & Jankun J (1998). Expression of the plasminogen activation system in kidney cancer correlates with its aggressive phenotype. *Clin Cancer Res* **4,** 869-877.

Syn WK, Jung Y, Omenetti A, Abdelmalek M, Guy CD, Yang L, Wang J, Witek RP, Fearing CM, Pereira TA, Teaberry V, Choi SS, Conde-Vancells J, Karaca GF & Diehl AM (2009). Hedgehog-mediated epithelial-to-mesenchymal transition and fibrogenic repair in nonalcoholic fatty liver disease. *Gastroenterology* **137,** 1478-1488.

Tache Y (2012). Brainstem neuropeptides and vagal protection of the gastric mucosal against injury: role of prostaglandins, nitric oxide and calcitonin-gene related peptide in capsaicin afferents. *Curr Med Chem* **19,** 35-42.

Takahashi M, Ota S, Nishimura S, Ogura K, Maeda S, Toda N, Hamada E, Terano A & Omata M (1996). Keratinocyte growth factor is an endogenous stimulant of rabbit gastric epithelial cell proliferation and migration in primary culture. *J Gastroenterol Hepatol* **11,** 1089-1096.

Takaishi S, Tu S, Dubeykovskaya ZA, Whary MT, Muthupalani S, Rickman BH, Rogers AB, Lertkowit N, Varro A, Fox JG & Wang TC (2009). Gastrin is an essential cofactor for Helicobacter-associated gastric corpus carcinogenesis in C57BL/6 mice. *Am J Pathol* **175,** 365-375.

Takeuchi K, Araki H, Umeda M, Komoike Y & Suzuki K (2001). Adaptive gastric cytoprotection is mediated by prostaglandin EP1 receptors: a study using rats and knockout mice. *J Pharmacol Exp Ther* **297,** 1160-1165.

Takeuchi K (2012). Pathogenesis of NSAID-induced gastric damage: importance of cyclooxygenase inhibition and gastric hypermotility. *World J Gastroenterol* **18,** 2147-2160.

Takezono Y, Joh T, Oshima T, Suzuki H, Seno K, Yokoyama Y, Alexander JS & Itoh M (2004). Role of prostaglandins in maintaining gastric mucus-cell permeability against acid exposure. *J Lab Clin Med* **143,** 52-58.

Tanaka A, Araki H, Hase S, Komoike Y & Takeuchi K (2002). Up-regulation of COX-2 by inhibition of COX-1 in the rat: a key to NSAID-induced gastric injury. *Aliment Pharmacol Ther* **16 Suppl 2,** 90-101.

Tanaka K, Nishimoto K, Tomisato W, Tsutsumi S, Hoshino T, Tsuchiya T & Mizushima T (2004). Adaptive cytoprotection induced by pretreatment with ethanol protects against gastric cell damage by NSAIDs. *Dig Dis Sci* **49,** 210-217.

Tanaka N, Fukao H, Ueshima S, Okada K, Yasutomi M & Matsuo O (1991). Plasminogen activator inhibitor 1 in human carcinoma tissues. *Int J Cancer* **48,** 481-484.

Tang H, Kerins DM, Hao Q, Inagami T & Vaughan DE (1998). The urokinase-type plasminogen activator receptor mediates tyrosine phosphorylation of focal adhesion proteins and activation of mitogen-activated protein kinase in cultured endothelial cells. *J Biol Chem* **273,** 18268-18272.

Tani T, Hanasawa K, Kodama M, Imaizumi H, Yonekawa M, Saito M, Ikeda T, Yagi Y, Takayama K, Amano I, Shimaoka H, Ohta M, Okahisa T, Koga N, Fujita N & Yamasa H (2001). Correlation between plasma endotoxin, plasma cytokines, and plasminogen activator inhibitor-1 activities in septic patients. *World J Surg* **25,** 660-668.

Tari A, Kamiyasu T, Yonei Y, Hamada M, Sumii M, Sumii K, Kajiyama G & Dimaline R (1997). Role of gastrin/CCK-B receptor in the regulation of gastric acid secretion in rat. *Dig Dis Sci* **42,** 1901-1907.

Tarnawski A, Hollander D, Stachura J, Krause WJ & Gergely H (1985). Prostaglandin protection of the gastric mucosa against alcohol injury--a dynamic time-related process. Role of the mucosal proliferative zone. *Gastroenterology* **88,** 334-352.

Tarnawski AS & Jones MK (1998). The role of epidermal growth factor (EGF) and its receptor in mucosal protection, adaptation to injury, and ulcer healing: involvement of EGF-R signal transduction pathways. *J Clin Gastroenterol* **27 Suppl 1,** S12-S20.

Tarnawski AS (2005). Cellular and molecular mechanisms of gastrointestinal ulcer healing. *Dig Dis Sci* **50 Suppl 1,** S24-S33.

Tarnawski AS & Ahluwalia A (2012). Molecular mechanisms of epithelial regeneration and neovascularization during healing of gastric and esophageal ulcers. *Curr Med Chem* **19,** 16-27.

Tarnawski AS, Ahluwalia A & Jones MK (2012). The mechanisms of gastric mucosal injury: focus on microvascular endothelium as a key target. *Curr Med Chem* **19,** 4-15.

Tarnawski A, Ahluwalia A & Jones MK (2013). Gastric cytoprotection beyond prostaglandins: cellular and molecular mechanisms of gastroprotective and ulcer healing actions of antacids. *Curr Pharm Des* **19,** 126-132.

Tatsuguchi A, Sakamoto C, Wada K, Akamatsu T, Tsukui T, Miyake K, Futagami S, Kishida T, Fukuda Y, Yamanaka N & Kobayashi M (2000). Localisation of cyclooxygenase 1 and cyclooxygenase 2 in Helicobacter pylori related gastritis and gastric ulcer tissues in humans. *Gut* **46,** 782-789.

Tétreault MP, Chailler P, Rivard N & Ménard D (2005). Differential growth factor induction and modulation of human gastric epithelial regeneration. *Exp Cell Res* **306,** 285-297.

Thomas DM, Nasim MM, Gullick WJ & Alison MR (1992). Immunoreactivity of transforming growth factor alpha in the normal adult gastrointestinal tract. *Gut* **33,** 628-631.

Thompson LJ, Danon SJ, Wilson JE, O'Rourke JL, Salama NR, Falkow S, Mitchell H & Lee A (2004). Chronic Helicobacter pylori infection with Sydney strain 1 and a newly identified mouse-adapted strain (Sydney strain 2000) in C57BL/6 and BALB/c mice. *Infect Immun* **72,** 4668-4679.

Thompson M, Fleming KA, Evans DJ, Fundele R, Surani MA & Wright NA (1990). Gastric endocrine cells share a clonal origin with other gut cell lineages. *Development* **110,** 477-481.

Tolhurst G, Reimann F & Gribble FM (2012). Intestinal sensing of nutrients. *Handb Exp Pharmacol* **209,** 309-355.

Toller IM, Hitzler I, Sayi A & Mueller A (2010). Prostaglandin E2 prevents Helicobacter-induced gastric preneoplasia and facilitates persistent infection in a mouse model. *Gastroenterology* **138,** 1455-1467.

Tomasek JJ, Gabbiani G, Hinz B, Chaponnier C & Brown RA (2002). Myofibroblasts and mechano-regulation of connective tissue remodelling. *Nat Rev Mol Cell Biol* **3,** 349-363.

Touati E, Michel V, Thiberge JM, Wuscher N, Huerre M & Labigne A (2003). Chronic Helicobacter pylori infections induce gastric mutations in mice. *Gastroenterology* **124,** 1408-1419.

Tramèr MR, Moore RA, Reynolds DJ & McQuay HJ (2000). Quantitative estimation of rare adverse events which follow a biological progression: a new model applied to chronic NSAID use. *Pain* **85,** 169-182.

Tsuchiya H, Sunayama C, Okada G, Matsuda E, Tomita K & Binder BR (1997). Plasminogen activator inhibitor-1 accelerates lung metastasis formation of human fibrosarcoma cells. *Anticancer Res* **17,** 313-316.

Tsutsui S, Shinomura Y, Higashiyama S, Higashimoto Y, Miyazaki Y, Kanayama S, Hiraoka S, Minami T, Kitamura S, Murayama Y, Miyagawa J, Taniguchi N & Matsuzawa Y (1997). Induction of heparin binding epidermal growth factor-like growth factor and amphiregulin mRNAs by gastrin in the rat stomach. *Biochem Biophys Res Commun* **235,** 520-523.

Tsutsumi R, Takahashi A, Azuma T, Higashi H & Hatakeyama M (2006). Focal adhesion kinase is a substrate and downstream effector of SHP-2 complexed with Helicobacter pylori CagA. *Mol Cell Biol* **26,** 261-276.

Tsutsumi S, Mima S, Tomisato W, Hoshino T, Tsuchiya T & Mizushima T (2003). Molecular mechanism of adaptive cytoprotection induced by ethanol in human gastric cells. *Exp Biol Med* **228,**1089-1095.

Tuan TL, Hwu P, Ho W, Yiu P, Chang R, Wysocki A & Benya PD (2008). Adenoviral overexpression and small interfering RNA suppression demonstrate that plasminogen activator inhibitor-1 produces elevated collagen accumulation in normal and keloid fibroblasts. *Am J Pathol* **173,** 1311-1325.

Tzaneva MA (2003). Ultrastructural immunohistochemical localization of gastrin, somatostatin and serotonin in endocrine cells of human antral gastric mucosa. *Acta Histochem* **105,** 191-201.

Uc A, Zhu X, Wagner BA, Buettner GR & Berg DJ (2012). Heme oxygenase-1 is protective against nonsteroidal anti-inflammatory drug-induced gastric ulcers. *J Pediatr Gastroenterol Nutr* **54,** 471-476.

Uemura N, Okamoto S, Yamamoto S, Matsumura N, Yamaguchi S, Yamakido M, Taniyama K, Sasaki N & Schlemper RJ (2001). Helicobacter pylori infection and the development of gastric cancer. *N Engl J Med* **345,** 784-789.

Umeda M, Murata-Kamiya N, Saito Y, Ohba Y, Takahashi M & Hatakeyama M (2009). Helicobacter pylori CagA causes mitotic impairment and induces chromosomal instability. *J Biol Chem* **284,** 22166-22172.

Vaday GG & Lider O (2000). Extracellular matrix moieties, cytokines, and enzymes: dynamic effects on immune cell behavior and inflammation. *J Leukoc Biol* **67,** 149-159.

Valentich JD & Powell DW (1994). Intestinal subepithelial myofibroblasts and mucosal immunophysiology. *Curr Opin Gastroenterol* **10,** 645-651.

Valnes K, Brandtzaeg P, Elgjo K & Stave R (1984). Specific and nonspecific humoral defense factors in the epithelium of normal and inflamed gastric mucosa. Immunohistochemical localization of immunoglobulins, secretory component, lysozyme, and lactoferrin. *Gastroenterology* **86,** 402-412.

van den Brink GR, Hardwick JC, Tytgat GN, Brink MA, Ten Kate FJ, Van Deventer SJ & Peppelenbosch MP (2001). Sonic hedgehog regulates gastric gland morphogenesis in man and mouse. *Gastroenterology* **121,** 317-328.

van den Borne SW, Diez J, Blankesteijn WM, Verjans J, Hofstra L & Narula J (2010). Myocardial remodeling after infarction: the role of myofibroblasts. *Nat Rev Cardiol* **7,** 30-37.

van der Burg JM, Winqvist A, Aziz NA, Maat-Schieman ML, Roos RA, Bates GP, Brundin P, Björkqvist M & Wierup N (2011). Gastrointestinal dysfunction contributes to weight loss in Huntington's disease mice. *Neurobiol Dis* **44,** 1-8.

van Rees BP, Saukkonen K, Ristimäki A, Polkowski W, Tytgat GN, Drillenburg P & Offerhaus GJ (2002). Cyclooxygenase-2 expression during carcinogenesis in the human stomach. *J Pathol* **196,** 171-179.

Vane JR (1971). Inhibition of prostaglandin synthesis as a mechanism of action for aspirin-like drugs. *Nat New Biol* **231,** 232-235.

Varro A, Noble PJ, Pritchard DM, Kennedy S, Hart CA, Dimaline R & Dockray GJ (2004). Helicobacter pylori induces plasminogen activator inhibitor 2 in gastric epithelial cells through nuclear factor-kappaB and RhoA: implications for invasion and apoptosis. *Cancer Res* **64,** 1695-1702.

Varro A, Kenny S, Hemers E, McCaig C, Przemeck S, Wang TC, Bodger K & Pritchard DM (2007). Increased gastric expression of MMP-7 in hypergastrinemia and significance for epithelial-mesenchymal signaling. *Am J Physiol Gastrointest Liver Physiol* **292,** G1133-G1140.

Veijola LI, Oksanen AM, Sipponen PI & Rautelin HI (2010). Association of autoimmune type atrophic corpus gastritis with Helicobacter pylori infection. *World J Gastroenterol* **16,** 83-88.

Vermeulen L, De Sousa E Melo F, van der Heijden M, Cameron K, de Jong JH, Borovski T, Tuynman JB, Todaro M, Merz C, Rodermond H, Sprick MR, Kemper K, Richel DJ, Stassi G & Medema JP (2010). Wnt activity defines colon cancer stem cells and is regulated by the microenvironment. *Nat Cell Biol* **12,** 468-476.

Vial E, Sahai E & Marshall CJ (2003). ERK-MAPK signaling coordinately regulates activity of Rac1 and RhoA for tumor cell motility. *Cancer Cell* **4,** 67-79.

Viala J, Chaput C, Boneca IG, Cardona A, Girardin SE, Moran AP, Athman R, Mémet S, Huerre MR, Coyle AJ, DiStefano PS, Sansonetti PJ, Labigne A, Bertin J, Philpott DJ & Ferrero RL (2004). Nod1 responds to peptidoglycan delivered by the Helicobacter pylori cag pathogenicity island. *Nat Immunol* **5,** 1166-1174.

von Montfort C, Beier JI, Kaiser JP, Guo L, Joshi-Barve S, Pritchard MT, States JC & Arteel GE (2010). PAI-1 plays a protective role in CCl4-induced hepatic fibrosis in mice: role of hepatocyte division. *Am J Physiol Gastrointest Liver Physiol* **298,** G657-G666.

Vreeburg EM, Levi M, Rauws EA, Deventer SJ, Snel P, Bartelsman JW, Ten Cate JW & Tytgat GN (2001). Enhanced mucosal fibrinolytic activity in gastroduodenal ulcer haemorrhage and the beneficial effect of acid suppression. *Aliment Pharmacol Ther* **15,** 639-646.

Wallace JL & Whittle BJ (1986a). The role of extracellular mucus as a protective cap over gastric mucosal damage. *Scand J Gastroenterol* **21 Suppl 125,** 79-85.

Wallace JL & Whittle BJ (1986b). Role of mucus in the repair of gastric epithelial damage in the rat. Inhibition of epithelial recovery by mucolytic agents. *Gastroenterology* **91,** 603-611.

Wallace JL (1988). Increased resistance of the rat gastric mucosa to hemorrhagic damage after exposure to an irritant. Role of the "mucoid cap" and prostaglandin synthesis. *Gastroenterology* **94,** 22-32.

Wallace JL & McKnight GW (1990). The mucoid cap over superficial gastric damage in the rat. A high-pH microenvironment dissipated by nonsteroidal antiinflammatory drugs and endothelin. *Gastroenterology* **99,** 295-304.

Wallace JL, McKnight W, Reuter BK & Vergnolle N (2000). NSAID-induced gastric damage in rats: requirement for inhibition of both cyclooxygenase 1 and 2. *Gastroenterology* **119,** 706-714.

Wallace JL & Miller MJ (2000). Nitric oxide in mucosal defense: a little goes a long way. *Gastroenterology* **119,** 512-520.

Wallace JL (2008). Prostaglandins, NSAIDs and gastric mucosal protection: why doesn’t the stomach digest itself? *Physiol Rev* **88,** 1547-1565.

Wallasch C, Crabtree JE, Bevec D, Robinson PA, Wagner H & Ullrich A (2002). Helicobacter pylori-stimulated EGF receptor transactivation requires metalloprotease cleavage of HB-EGF. *Biochem Biophys Res Commun* **295,** 695-701.

Waltz DA, Natkin LR, Fujita RM, Wei Y & Chapman HA (1997). Plasmin and plasminogen activator inhibitor type 1 promote cellular motility by regulating the interaction between the urokinase receptor and vitronectin. *J Clin Invest* **100,** 58-67.

Wang H, Vohra BP, Zhang Y & Heuckeroth RO (2005). Transcriptional profiling after bile duct ligation identifies PAI-1 as a contributor to cholestatic injury in mice. *Hepatology* **42,** 1099-1108.

Wang TC, Koh TJ, Varro A, Cahill RJ, Dangler CA, Fox JG & Dockray GJ (1996). Processing and proliferative effects of human progastrin in transgenic mice. *J Clin Invest* **98,** 1918-1929.

Wang TC, Goldenring JR, Dangler C, Ito S, Mueller A, Jeon WK, Koh TJ & Fox JG (1998). Mice lacking secretory phospholipase A2 show altered apoptosis and differentiation with Helicobacter felis infection. *Gastroenterology* **114,** 675-689.

Wang TC, Dangler CA, Chen D, Goldenring JR, Koh T, Raychowdhury R, Coffey RJ, Ito S, Varro A, Dockray GJ & Fox JG (2000). Synergistic interaction between hypergastrinemia and Helicobacter infection in a mouse model of gastric cancer. *Gastroenterology* **118,** 36-47.

Wang YH, Taché Y, Sheibel AB, Go VL & Wei JY (1997). Two types of leptin-responsive gastric vagal afferent terminals: an in vitro single-unit study in rats. *Am J Physiol Regul Integr Comp Physiol* **273,** R833-R837.

Wang Z, Zhao Q, Han Y, Zhang D, Zhang L & Luo D (2013). PAI-1 and IFN-γ in the regulation of innate immune homeostasis during sublethal yersiniosis. *Blood Cells Mol Dis* **50,** 196-201.

Watanabe S, Hirose M, Wang XE, Kobayashi O, Nagahara A, Murai T, Iwazaki R, Miwa H, Miyazaki A & Sato N (2000). Epithelial-mesenchymal interaction in gastric mucosal restoration. *J Gastroenterol* **35 Suppl 12,** 65-68.

Watson SA, Grabowska AM, El-Zaatari M & Takhar A (2006). Gastrin - active participant or bystander in gastric carcinogenesis? *Nat Rev Cancer* **6,** 936-946.

Weeks DL, Eskandari S, Scott DR & Sachs G (2000). A H+-gated urea channel: the link between Helicobacter pylori urease and gastric colonization. *Science* **287,** 482-485.

Wei Y, Waltz DA, Rao N, Drummond RJ, Rosenberg S & Chapman HA (1994). Identification of the urokinase receptor as an adhesion receptor for vitronectin. *J Biol Chem* **269,** 32380-32388.

Wei Y, Lukashev M, Simon DI, Bodary SC, Rosenberg S, Doyle MV & Chapman HA (1996). Regulation of integrin function by the urokinase receptor. *Science* **273,** 1551-1555.

Wei Y, Yang X, Liu Q, Wilkins JA & Chapman HA (1999). A role for caveolin and the urokinase receptor in integrin-mediated adhesion and signaling. *J Cell Biol* **144,** 1285-1294.

Wei Y, Eble JA, Wang Z, Kreidberg JA & Chapman HA (2001). Urokinase receptors promote beta1 integrin function through interactions with integrin alpha3beta1. *Mol Biol Cell* **12,** 2975-2986.

Wei Y, Czekay RP, Robillard L, Kugler MC, Zhang F, Kim KK, Xiong JP, Humphries MJ & Chapman HA (2005). Regulation of alpha5beta1 integrin conformation and function by urokinase receptor binding. *J Cell Biol* **168,** 501-511.

Weis VG & Goldenring JR (2009). Current understanding of SPEM and its standing in the preneoplastic process. *Gastric Cancer* **12,** 189-197.

Wellstein A, Fang WJ, Khatri A, Lu Y, Swain SS, Dickson RB, Sasse J, Riegel AT & Lippman ME (1992). A heparin-binding growth factor secreted from breast cancer cells homologous to a developmentally regulated cytokine. *J Biol Chem* **267,** 2582-2587.

Werner S & Grose R (2003). Regulation of wound healing by growth factors and cytokines. *Physiol Rev* **83,** 835-870.

Whitelock JM, Murdoch AD, Iozzo RV & Underwood PA (1996). The degradation of human endothelial cell-derived perlecan and release of bound basic fibroblast growth factor by stromelysin, collagenase, plasmin, and heparanases, *J Biol Chem* **271,** 10079-10086.

Whittle BJ (1981). Arachidonic acid metabolites and the gastro-intestinal toxicity of anti-inflammatory agents. *Prostaglandins* **21 Suppl,** 113-118.

Willems G, Vansteenkiste Y & Limbosch JM (1972). Stimulating effect of gastrin on cell proliferation kinetics in canine fundic mucosa. *Gastroenterology* **62,** 583-589.

Wiman B, Almquist A, Sigurdardottir O & Lindahl T (1988). Plasminogen activator inhibitor 1 (PAI) is bound to vitronectin in plasma. *FEBS Lett* **242,** 125-128.

Wipff PJ, Rifkin DB, Meister JJ & Hinz B (2007). Myofibroblast contraction activates latent TGF-beta1 from the extracellular matrix. *J Cell Biol* **179,** 1311-1323.

Wodziński MA, Bardhan KD, Reilly JT, Cooper P & Preston FE (1993). Reduced tissue type plasminogen activator activity of the gastroduodenal mucosa in peptic ulcer disease. *Gut* **34,** 1310-1314.

Wong AP, Cortez SL & Baricos WH (1992). Role of plasmin and gelatinase in extracellular matrix degradation by cultured rat mesangial cells. *Am J Physiol Renal Physiol* **263**, F1112-F1118.

Worthley DL, Ruszkiewicz A, Davies R, Moore S, Nivison-Smith I, Bik To L, Browett P, Western R, Durrant S, So J, Young GP, Mullighan CG, Bardy PG & Michael MZ (2009). Human gastrointestinal neoplasia-associated myofibroblasts can develop from bone marrow-derived cells following allogeneic stem cell transplantation. *Stem Cells* **27,** 1463-1468.

Wright NA, Pike CM & Elia G (1990). Ulceration induces a novel epidermal growth factor-secreting cell lineage in human gastrointestinal mucosa. *Digestion* **46 Suppl 2,** 125-133.

Wroblewski LE, Noble PJ, Pagliocca A, Pritchard DM, Hart CA, Campbell F, Dodson AR, Dockray GJ & Varro A (2003). Stimulation of MMP-7 (matrilysin) by Helicobacter pylori in human gastric epithelial cells: role in epithelial cell migration. *J Cell Sci* **116,** 3017-3026.

Wroblewski LE, Peek R M Jr & Wilson KT (2010) Helicobacter pylori and gastric cancer: factors that modulate disease risk *Clin Microbiol Rev* **23,** 713-739.

Wu J, Peng L, McMahon GA, Lawrence DA & Fay WP (2009). Recombinant plasminogen activator inhibitor-1 inhibits intimal hyperplasia. *Arterioscler Thromb Vasc Biol* **29,** 1565-1570.

Wu KC, Jackson LM, Galvin AM, Gray T, Hawkey CJ & Mahida YR (1999). Phenotypic and functional characterisation of myofibroblasts, macrophages, and lymphocytes migrating out of the human gastric lamina propria following the loss of epithelial cells. *Gut* **44,** 323-330.

Wu MS, Lin YS, Chang YT, Shun CT, Lin MT & Lin JT (2005). Gene expression profiling of gastric cancer by microarray combined with laser capture microdissection. *World J Gastroenterol* **11,** 7405-7412.

Xiang G, Schuster MD, Seki T, Witkowski P, Eshghi S & Itescu S (2005). Downregulated expression of plasminogen activator inhibitor-1 augments myocardial neovascularization and reduces cardiomyocyte apoptosis after acute myocardial infarction. *J Am Coll Cardiol* **46,** 536-541.

Xiao C, Ogle SA, Schumacher MA, Orr-Asman MA, Miller ML, Lertkowit N, Varro A, Hollande F & Zavros Y (2010). Loss of parietal cell expression of Sonic hedgehog induces hypergastrinemia and hyperproliferation of surface mucous cells. *Gastroenterology* **138,** 550-561.

Xu Z, Balsara RD, Gorlatova NV, Lawrence DA, Castellino FJ & Ploplis VA (2004). Conservation of critical functional domains in murine plasminogen activator inhibitor-1. *J Biol Chem* **279,** 17914-17920.

Yamaguchi I, Lopez-Guisa JM, Cai X, Collins SJ, Okamura DM & Eddy AA (2007). Endogenous urokinase lacks antifibrotic activity during progressive renal injury. *Am J Physiol Renal Physiol* **293,** F12-F19.

Yamaoka Y, Kwon DH & Graham DY (2000). A M(r) 34,000 proinflammatory outer membrane protein (oipA) of Helicobacter pylori. *Proc Natl Acad Sci U S A* **97,** 7533-7538.

Yamaoka Y, Kikuchi S, el-Zimaity HM, Gutierrez O, Osato MS & Graham DY (2002). Importance of Helicobacter pylori oipA in clinical presentation, gastric inflammation, and mucosal interleukin 8 production. *Gastroenterology* **123,** 414-424.

Yoo JK, Murakami M, Teramura S, Saita H, Kusaka S, Matsuo K, Kita T & Miyake T (1989). Effect of plasmin inhibitors on gastric mucosal permeability in rats. *Scand J Gastroenterol* **24 Suppl 162,** 215-217.

Yu W, Kim J & Ossowski L (1997) Reduction in surface urokinase receptor forces malignant cells into a protracted state of dormancy. *J Cell Biol* **137,** 767-777.

Zavadil J, Cermak L, Soto-Nieves N & Böttinger EP (2004). Integration of TGF-beta/Smad and Jagged1/Notch signalling in epithelial-to-mesenchymal transition. *EMBO J* **23,** 1155-1165.

Zhang G, Kim H, Cai X, Lopez-Guisa J, Alpers C, Liu Y, Carmeliet P & Eddy A (2003a). Urokinase receptor deficiency accelerates fibrosis in obstructive nephropathy. *J Am Soc Nephrol* **14,** 1254-1271.

Zhang G, Kim H, Cai X, Lopez-Guisa J, Carmeliet P & Eddy A (2003b). Urokinase receptor modulates cellular and angiogenic responses in obstructive uropathy. *J Am Soc Nephrol* **14,** 1254-1271.

Zhang G & Eddy AA (2008). Urokinase and its receptors in chronic kidney disease. *Front Biosci* **13,** 5462-5478.

Zhang L, Zhao ZS, Ru GQ & Ma J (2006). Correlative studies on uPA mRNA and uPAR mRNA expression with vascular endothelial growth factor, microvessel density, progression and survival time of patients with gastric cancer. *World J Gastroenterol* **12,** 3970-3976.

Zhang YP, Wang WL, Liu J, Li WB, Bai LL, Yuan YD & Song SX (2013). Plasminogen activator inhibitor-1 promotes the proliferation and inhibits the apoptosis of pulmonary fibroblasts by Ca(2+) signaling. *Thromb Res* **131,** 64-71.

Zmijewski JW, Bae HB, Deshane JS, Peterson CB, Chaplin DD & Abraham E (2011). Inhibition of neutrophil apoptosis by PAI-1. *Am J Physiol Lung Cell Mol Physiol* **301,** L247-L254.

Zorio E, Gilabert-Estellés J, España F, Ramón LA, Cosín R & Estellés A (2008). Fibrinolysis: the key to new pathogenetic mechanisms. *Curr Med Chem* **15,** 923-929.

APPENDIX

Affymetrix ID	Gene Symbol	Gene Title	Fold-change (PAI-1$^{-/-}$: C57BL/6)
1434484_at	1100001G20 Rik	RIKEN cDNA 1100001G20 gene	-6
1460049_s_at	1500015O10 Rik	RIKEN cDNA 1500015O10 gene	-67
1456158_at	2610109H07 Rik	RIKEN cDNA 2610109H07 gene	-9
1427054_s_at	Abi3bp	ABI gene family, member 3 (NESH) binding protein	-40
1421171_at	Adam12	a disintegrin and metallopeptidase domain 12 (meltrin alpha)	-61
1447946_at	Adam23	a disintegrin and metallopeptidase domain 23	-5
1444628_at	Adam33	a disintegrin and metallopeptidase domain 33	4
1419476_at	Adamdec1	ADAM-like, decysin 1	4
1456901_at	Adamts20	a disintegrin-like and metallopeptidase (reprolysin type) with thrombospondin type 1 motif, 20	-15
1452595_at	Adamts4	a disintegrin-like and metallopeptidase (reprolysin type) with thrombospondin type 1 motif, 4	-3
1437785_at	Adamts9	a disintegrin-like and metallopeptidase (reprolysin type) with thrombospondin type 1 motif, 9	-8
1430313_at	Adamtsl1	ADAMTS-like 1	-93
1417765_a_at	Amy1	amylase 1, salivary	-4
1439066_at	Angpt1	angiopoietin 1	-6
1455224_at	Angptl1	angiopoietin-like 1	-8
1417130_s_at	Angptl4	angiopoietin-like 4	-3
1451478_at	Angptl7	angiopoietin-like 7	8
1416371_at	Apod	apolipoprotein D	-4
1432466_a_at	Apoe	apolipoprotein E	-5
1427996_at	BC028528	cDNA sequence BC028528	-13
1422168_a_at	Bdnf	brain derived neurotrophic factor	13
1422912_at	Bmp4	bone morphogenetic protein 4	7
1450759_at	Bmp6	bone morphogenetic protein 6	10
1429273_at	Bmper	BMP-binding endothelial regulator	3
1435541_at	Btc	betacellulin, epidermal growth factor family member	4
1451620_at	C1ql3	C1q-like 3	5
1431079_at	C1qtnf2	C1q and tumor necrosis factor related protein 2	8
1422606_at	C1qtnf3	C1q and tumor necrosis factor related protein 3	-47
1417050_at	C1qtnf4	C1q and tumor necrosis factor related protein 4	-4

1431856_a_at	C1qtnf6	C1q and tumor necrosis factor related protein 6	-3
1416051_at	C2	complement component 2 (within H-2S)	15
1423954_at	C3	complement component 3	-7
1418021_at	C4b	complement component 4B (Childo blood group)	-6
1418037_at	C4bp	complement component 4 binding protein	28
1451625_a_at	C8g	complement component 8, gamma polypeptide	-6
1452004_at	Calca	calcitonin/calcitonin-related polypeptide, alpha	209
1422639_at	Calcb	calcitonin-related polypeptide, beta	3
1419691_at	Camp	cathelicidin antimicrobial peptide	-9
1419473_a_at	Cck	cholecystokinin	13
1417789_at	Ccl11	chemokine (C-C motif) ligand 11	-35
1420380_at	Ccl2	chemokine (C-C motif) ligand 2	-11
1418126_at	Ccl5	chemokine (C-C motif) ligand 5	-39
1421228_at	Ccl7	chemokine (C-C motif) ligand 7	-21
1419684_at	Ccl8	chemokine (C-C motif) ligand 8	-9
1417936_at	Ccl9	chemokine (C-C motif) ligand 9	-102
1417314_at	Cfb	complement factor B	-8
1450876_at	Cfh /// LOC100048018	complement component factor h /// similar to complement component factor H	5
1434201_at	Chrdl1	chordin-like 1	3
1425769_x_at	Cklf	chemokine-like factor	-3
1418980_a_at	Cnp	2',3'-cyclic nucleotide 3' phosphodiesterase	-3
1418599_at	Col11a1	collagen, type XI, alpha 1	-5
1446326_at	Col1a2	collagen, type I, alpha 2	-6
1448590_at	Col6a1	collagen, type VI, alpha 1	-9
1452250_a_at	Col6a2	collagen, type VI, alpha 2	-32
1424131_at	Col6a3	collagen, type VI, alpha 3	-25
1434667_at	Col8a2	collagen, type VIII, alpha 2	-6
1448735_at	Cp	ceruloplasmin	-4
1460248_at	Cpxm2	carboxypeptidase X 2 (M14 family)	9
1426951_at	Crim1	cysteine rich transmembrane BMP regulator 1 (chordin like)	4
1437056_x_at	Crispld2	cysteine-rich secretory protein LCCL domain containing 2	-19
1425155_x_at	Csf1	colony stimulating factor 1 (macrophage)	6
1416953_at	Ctgf	connective tissue growth factor	27
1452968_at	Cthrc1	collagen triple helix repeat containing 1	-68
1453282_at	Cxadr	coxsackie virus and adenovirus receptor	3
1419209_at	Cxcl1	chemokine (C-X-C motif) ligand 1	-4
1418930_at	Cxcl10	chemokine (C-X-C motif) ligand 10	-9
1417574_at	Cxcl12	chemokine (C-X-C motif) ligand 12	-8
1421404_at	Cxcl15	chemokine (C-X-C motif) ligand 15	-21
1449984_at	Cxcl2	chemokine (C-X-C motif) ligand 2	-7
1419728_at	Cxcl5	chemokine (C-X-C motif) ligand 5	-145
1442340_x_at	Cyr61	cysteine rich protein 61	-3
1416697_at	Dpp4	dipeptidylpeptidase 4	109

1418511_at	Dpt	dermatopontin	-126
1448613_at	Ecm1	extracellular matrix protein 1	3
1433474_at	Edil3	EGF-like repeats and discoidin I-like domains 3	-7
1419332_at	Egfl6	EGF-like-domain, multiple 6	-61
1426541_a_at	Endod1	endonuclease domain containing 1	4
1436799_at	Enox1	ecto-NOX disulfide-thiol exchanger 1	-5
1448136_at	Enpp2	ectonucleotide pyrophosphatase/phosphodiesterase 2	-120
1439260_a_at	Enpp3	ectonucleotide pyrophosphatase/phosphodiesterase 3	-76
1421114_a_at	Epyc	epiphycan	-6
1434606_at	Erbb3	v-erb-b2 erythroblastic leukemia viral oncogene homolog 3 (avian)	5
1419431_at	Ereg	epiregulin	6
1417688_at	Fam20c	family with sequence similarity 20, member C	3
1460251_at	Fas	Fas (TNF receptor superfamily member 6)	3
1422540_at	Fbln1	fibulin 1	-20
1438375_at	Fbln2	Fibulin 2, mRNA (cDNA clone MGC:6112 IMAGE:3490759)	4
1460412_at	Fbln7	fibulin 7	-3
1449545_at	Fgf18	fibroblast growth factor 18	6
1449826_a_at	Fgf2	fibroblast growth factor 2	6
1422916_at	Fgf21	fibroblast growth factor 21	-5
1438405_at	Fgf7	fibroblast growth factor 7	-14
1438718_at	Fgf9	fibroblast growth factor 9	23
1420847_a_at	Fgfr2	fibroblast growth factor receptor 2	-4
1424599_at	Fgl1	fibrinogen-like protein 1	-6
1456084_x_at	Fmod	fibromodulin	-29
1437218_at	Fn1	fibronectin 1	3
1455280_at	Frem1	Fras1 related extracellular matrix protein 1	-8
1416658_at	Frzb	frizzled-related protein	9
1421365_at	Fst	follistatin	4
1418949_at	Gdf15	growth differentiation factor 15	3
1419080_at	Gdnf	glial cell line derived neurotrophic factor	3
1424927_at	Glipr1	GLI pathogenesis-related 1 (glioma)	17
1457429_s_at	Gm106	gene model 106, (NCBI)	-31
1429293_at	Gpc2	glypican 2 (cerebroglycan)	-7
1417836_at	Gpx7	glutathione peroxidase 7	-4
1418492_at	Grem2	gremlin 2 homolog, cysteine knot superfamily (Xenopus laevis)	-3
1433930_at	Hpse	heparanase	-8
1416749_at	Htra1	HtrA serine peptidase 1	4
1419292_at	Htra3	HtrA serine peptidase 3	-23
1440169_x_at	Ifnar2	interferon (alpha and beta) receptor 2	-3
1454159_a_at	Igfbp2	insulin-like growth factor binding protein 2	9
1458268_s_at	Igfbp3	insulin-like growth factor binding protein 3	5
1421991_a_at	Igfbp4	insulin-like growth factor binding protein 4	-39
1452114_s_at	Igfbp5	insulin-like growth factor binding protein 5	-56

1427351_s_at	Igh-6	immunoglobulin heavy chain 6 (heavy chain of IgM)	21
1426566_s_at	Il17re	interleukin 17 receptor E	14
1417932_at	Il18	interleukin 18	3
1425145_at	Il1rl1	interleukin 1 receptor-like 1	-19
1416200_at	Il33	interleukin 33	4
1421034_a_at	Il4ra	interleukin 4 receptor, alpha	5
1422053_at	Inhba	inhibin beta-A	9
1426858_at	Inhbb /// LOC100046802	inhibin beta-B /// similar to Inhbb protein	16
1431591_s_at	Isg15	ISG15 ubiquitin-like modifier	-8
1418450_at	Islr	immunoglobulin superfamily containing leucine-rich repeat	-4
1429159_at	Itih5	inter-alpha (globulin) inhibitor H5	-6
1440955_at	Kcp	kielin/chordin-like protein	-3
1426152_a_at	Kitl	kit ligand	14
1419722_at	Klk8	kallikrein related-peptidase 8	31
1418153_at	Lama1	laminin, alpha 1	7
1426285_at	Lama2	laminin, alpha 2	5
1427512_a_at	Lama3	laminin, alpha 3	-8
1427747_a_at	Lcn2	lipocalin 2	-26
1448380_at	Lgals3bp	lectin, galactoside-binding, soluble, 3 binding protein	-3
1440147_at	Lgi2	leucine-rich repeat LGI family, member 2	-19
1421207_at	Lif	leukemia inhibitory factor	17
1454984_at	Lifr	leukemia inhibitory factor receptor	-4
1450188_s_at	Lipg	lipase, endothelial	7
1448228_at	Lox	lysyl oxidase	4
1452436_at	Loxl2	lysyl oxidase-like 2	6
1415904_at	Lpl	lipoprotein lipase	-13
1423607_at	Lum	lumican	-7
1454969_at	Lypd6	LY6/PLAUR domain containing 6	-5
1455978_a_at	Matn2	matrilin 2	-6
1416006_at	Mdk	midkine	-30
1417359_at	Mfap2	microfibrillar-associated protein 2	-15
1449082_at	Mfap5	microfibrillar associated protein 5	20
1417234_at	Mmp11	matrix metallopeptidase 11	-4
1437568_at	Mmp16	matrix metallopeptidase 16	-5
1417282_at	Mmp23	matrix metallopeptidase 23	-5
1416298_at	Mmp9	matrix metallopeptidase 9	-11
1460238_at	Msln	mesothelin	71
1450468_at	Myoc	myocilin	-14
1426851_a_at	Nov	nephroblastoma overexpressed gene	-7
1440240_at	Npb	neuropeptide B	-15
1452107_s_at	Npnt	nephronectin	-18
1434802_s_at	Ntf3	neurotrophin 3	14
1450512_at	Ntn4	netrin 4	48
1436718_at	Nxph1	neurexophilin 1	58
1448475_at	Olfml3	olfactomedin-like 3	-7

1419534_at	Olr1	oxidized low density lipoprotein (lectin-like) receptor 1	28
1418745_at	Omd	osteomodulin	-4
1432591_at	Pappa	pregnancy-associated plasma protein A	17
1448433_a_at	Pcolce	procollagen C-endopeptidase enhancer protein	-4
1448995_at	Pf4	platelet factor 4	-35
1429019_s_at	Pon2	paraoxonase 2	3
1449824_at	Prg4	proteoglycan 4 (megakaryocyte stimulating factor, articular superficial zone protein)	-5
1417905_at	Prl7a2	prolactin family 7, subfamily a, member 2	34
1448608_at	Prl8a2	prolactin family 8, subfamily a, member 2	4
1439549_at	Prrg3	proline rich Gla (G-carboxyglutamic acid) 3 (transmembrane)	7
1446560_at	Prss23	protease, serine, 23	10
1416211_a_at	Ptn	pleiotrophin	-3667
1417346_at	Pycard	PYD and CARD domain containing	3
1426225_at	Rbp4	retinol binding protein 4, plasma	-3
1453215_at	Rnase10	ribonuclease, RNase A family, 10 (non-active)	-4
1449319_at	Rspo1	R-spondin homolog (Xenopus laevis)	156
1455893_at	Rspo2	R-spondin 2 homolog (Xenopus laevis)	-164
1419383_at	S100b	S100 protein, beta polypeptide, neural	-3
1450708_at	Scg2	secretogranin II	-688
1423150_at	Scg5	secretogranin V	-4
1420417_at	Sema3a	sema domain, immunoglobulin domain (Ig), short basic domain, secreted, (semaphorin) 3A	5
1427673_a_at	Sema3e	sema domain, immunoglobulin domain (Ig), short basic domain, secreted, (semaphorin) 3E	21
1460187_at	Sfrp1	secreted frizzled-related protein 1	-5
1448201_at	Sfrp2	secreted frizzled-related protein 2	-66
1448377_at	Slpi	secretory leukocyte peptidase inhibitor	23
1415935_at	Smoc2	SPARC related modular calcium binding 2	-13
1454710_at	Spink2	serine peptidase inhibitor, Kazal type 2	-8
1416627_at	Spint1	serine protease inhibitor, Kunitz type 1	11
1451935_a_at	Spint2	serine protease inhibitor, Kunitz type 2	14
1418076_at	St14	suppression of tumorigenicity 14 (colon carcinoma)	17
1420928_at	St6gal1	beta galactoside alpha 2,6 sialyltransferase 1	4
1436319_at	Sulf1	sulfatase 1	6
1430388_a_at	Sulf2	sulfatase 2	6
1418547_at	Tfpi2	tissue factor pathway inhibitor 2	9
1438303_at	Tgfb2	transforming growth factor, beta 2	4
1417455_at	Tgfb3	transforming growth factor, beta 3	3
1456250_x_at	Tgfbi	transforming growth factor, beta induced	-32
1433795_at	Tgfbr3	transforming growth factor, beta receptor III	-3
1450377_at	Thbs1	thrombospondin 1	3
1447862_x_at	Thbs2	thrombospondin 2	-71
1416623_at	Thbs3	thrombospondin 3	-8
1455262_at	Thsd4	thrombospondin, type I, domain containing 4	-4

1450040_at	Timp2	tissue inhibitor of metalloproteinase 2	4
1419088_at	Timp3	tissue inhibitor of metalloproteinase 3	7
1423405_at	Timp4	tissue inhibitor of metalloproteinase 4	-4
1417109_at	Tinagl1	tubulointerstitial nephritis antigen-like 1	21
1420753_at	Tll1	tolloid-like	6
1436644_x_at	Tmem25	transmembrane protein 25	-16
1456344_at	Tnc	Tenascin C (Tnc), mRNA	22
1449033_at	Tnfrsf11b	tumor necrosis factor receptor superfamily, member 11b (osteoprotegerin)	25
1448147_at	Tnfrsf19	tumor necrosis factor receptor superfamily, member 19	-82
1442590_at	Tnfrsf22 /// Tnfrsf23	tumor necrosis factor receptor superfamily, member 22 /// tumor necrosis factor receptor superfamily, member 23	3
1419083_at	Tnfsf11	tumor necrosis factor (ligand) superfamily, member 11	-136
1425546_a_at	Trf	transferrin	626
1416689_at	Tuft1	tuftelin 1	7
1427256_at	Vcan	versican	-4
1439766_x_at	Vegfc	vascular endothelial growth factor C	-4
1452065_at	Vstm2a	V-set and transmembrane domain containing 2A	126
1428811_at	Wfikkn2	WAP, follistatin/kazal, immunoglobulin, kunitz and netrin domain containing 2	-6
1448593_at	Wisp1	WNT1 inducible signaling pathway protein 1	4
1419015_at	Wisp2	WNT1 inducible signaling pathway protein 2	11
1422941_at	Wnt16	wingless-related MMTV integration site 16	-47
1436978_at	Wnt9a	wingless-type MMTV integration site 9A	10

Table A.1 All transcripts encoding extracellular proteins differentially expressed by cultured PAI-1$^{-/-}$ antral myofibroblasts compared to cultured wild type antral myofibroblasts. There were a total of 223 genes, classified as ‘extracellular’ by gene product ontology, that were significantly differentially expressed by PAI-1$^{-/-}$ antral myofibroblasts compared to wild type (unpaired *t*-test, Benjamini-Hochberg correction, $P < 0.05$, fold-change ≥ 3 or ≤ -3).